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Examination of Lectin-Carbohydrate interactions during T cell-APC conjugation and a potential role for CD45

by

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ABSTRACT

Both T cell development and activation rely not only on the binding of the TCR to its corresponding ligand, but also on the interactions of numerous accessory molecules. One such molecule is CD45, which although well characterized in terms of its cytoplasmic domain and phosphatase activity, little is known regarding its complex and strictly regulated extracellular domain. It has been proposed that its activity is regulated through binding of specific ligands to different forms of this molecule, likely through presented carbohydrates. To this end, we have characterized three different C-type lectin receptors all of which are expressed on professional APCs. Although binding appeared independent of CD45, two lectins, CIRE and Dectin-1, did recognize T cells and positively regulated their activity. Additionally, studies using antibodies revealed that binding of specific epitopes of CD45 can elicit significant and varied effects, implicating CD45-ligand interactions in the regulation of T cell development and activation.

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LIST OF ABBREVIATIONS

AP-1	Activator Protein-1
APC	Antigen presenting cell
ATCC	.merican Type Culture Collection
β_2m	β-2 microglobulin
β-ME	β-Mercaptoethanol
Bim-1	Bisindolymaleimide
BLT	Benzyloxy carbonyl-L-lysine Thiobenzyl Ester
BSA	Bovine Serum Albumin
CD	Cluster Designation
ConA	Concanavalin A
CRD	Carbohydrate Recognition Domain
Csk	C-terminal Src-family kinase
CTL	Cytotoxic lymphocyte
CTLD	C-type lectin-like domain
CytoE	Cytochalasin E
D1	Membrane proximal CD45 phosphatase domain
D2	Membrane distal CD45 phosphatase domain
DC	Dendritic cell
DCAL-1	DC-associated lectin-1
DCIR	Dendritic cell immunoreceptor family of lectins
DCS	Defined Calf Serum
Dectin	DC-associated lectin
DC-SIGN	Dendritic cell specific ICAM-grabbing non-integrin
DMEM	Dulbecco's modified eagle medium
DN	Double Negative
DP	Double Positive
DTT	Dithiothreitol
ECC	Enhanced chemiluminescence
ECM	Extracellular Matrix
EDTA	Ethylenedinitrilo-tetraacetic acid
EGTA	Ethylene glycol bis(2-aminoethyl ether)-tetraacetic acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-linked Immunosorbent assay
ERK	Extracellular Regulated Kinase
FACS	Fluorescence activated cell sorting
FCS	Fetal Calf Serum
FITC	Fluorescence isothyocyanate
FTOC	Fetal Thymic Organ Culture

Fyn	Src-family kinase p59
GII	Glucosidase II
HRP	Horse Radish Peroxidase
ICAM	Intercellular Adhesion Molecule
ICOS	Inducible T-cell costimulator
IFN	Interferon
Ig	Immunogluobulin
IL	Interleukin
IP	Immunoprecipitate
IP3	Inositol Tris-phosphate
IPTG	Isopropyl-beta-D-thiogalactopyranoside
ITAM	Immuno-tyrosine based Activation Motif
JNK	Jun N-terminal Kinase
kDa	kilodalton
KO	Knock out
LAT	Linker for activation of T cells
LB	Luria-Bertani
Lck	Lymphocyte-specific Cytoplasmic protein Tyrosine Kinase
LFA	Leukocyte Function Associated Antigen
LL	Lower left quadrant
LR	Lower right quadrant
mAb	Monoclonal antibody
MACS	Magnetic Activated Cell Sorting
MAPK	Mitogen Activated Protein Kinase
MFI	Mean Fluorescence intensity
MHC	Major Histocompatability Complex
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor κ B
NK	Natural Killer
NKCL	Natural Killer C-type lectin
NTP	Nucleotide Tri-phosphate
OD	Optical Density
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PE	Phycoerythrin
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction

PDGFR	Platelet Derived Growth Factor Receptor
Pen/Strep.	Penicillin / streptomycin
PI3K	Phosphatidyl Inositol 3-Kinase
PLC-γ	Phospholipase C-γ
PMA	Phorbol-12 Myristate Acetate
Pre-Tα	Pre-TCR-α-chain
PTK	Protein tyrosine kinase
PTPase	Protein tyrosine phosphatase
Pyk2	Proline-rich tyrosine kinase 2
RPTP	Receptor-like Protein Tyrosine Phosphatases
RSB	Reducing Sample Buffer
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SCID	Severe Combined ImmunoDeficiency
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SFK	Src-Family Kinase
SH	Src-homology domain
SIGNR1	SIGN-related gene-1
SLP-76	SH2-domain containing leukocyte protein of 76 kDa
SP	Single Positive
TBS	Tris Buffered Saline
TCR	T cell Receptor
TEC	Thymic Epithelial Cell
T_H	T-helper Cell
TN	Triple Negative
TNF	Tumor Necrosis Factor
TSC	Thymic Stromal Cell
Unc-119	Uncoordinated-119
UL	Upper left quadrant
UR	Upper right quadrant
Y_{394}	Tyrosine 394 of Lck
Y_{505}	Tyrosine 505 of Lck
ZAP-70	ζ-associated protein of 70kDa

CHAPTER I

INTRODUCTION

1.1 Role of the Immune System

The immune system is a remarkably complex and adaptive defense system that has evolved in order to protect humans and other vertebrates from the plethora of pathogenic microorganisms that bombard us on a daily basis; while also providing protection from the development and spread of altered or transformed self cells (cancer). In doing so, this system must be capable of recognizing and eliminating an almost limitless variety of threats, yet retain the ability to differentiate between these and normal self to avoid autoimmunity.

1.1.1 Innate and Adaptive Branches of the Immune System

The immune system can be effectively separated into two major branches: the innate immune system and the adaptive immune system. Although these systems operate in a highly coordinated and often mutually dependent fashion, they also work on very different timelines and employ different mechanisms in the removal of that which is recognized as non-self. Innate immunity represents the first line of defense against foreign invaders, and comprises four primary barriers: Anatomic, physiologic, phagocytic and inflammatory. Anatomical barriers include mechanical barriers to pathogen entry such as skin and mucous membranes, whereas physiological barriers comprise of body temperature, low pH in the stomach, anti-microbial enzymes and proteins such as lysozyme, interferon and complement; all of which act to inhibit the growth and spread of microorganisms. In the situation that these first barriers are breeched or upon any tissue damage, a complex series of events, collectively referred to as inflammation, occurs.

This allows for increased blood flow into the affected area as well as increased vascular permeabilization in the vicinity of the injury, allowing for massive influx of phagocytic leukocytes including monocytes, macrophage, neutrophils and dendritic cells (DCs) from the bloodstream into the site of injury. These cells can then contribute, both directly and indirectly, to pathogen clearance. Directly, these cells phagocytose, kill and digest foreign pathogens. Indirectly, these cells, once having made contact with foreign microorganisms, will be triggered to release a variety of soluble mediators that will provide important information to the adaptive immune system as to what type of response is required.

Upon activation with proinflammatory stimuli, DCs express relatively high levels of class I and II major histocompatibility complex (MHC) proteins, in addition to upregulation of adhesion and co-stimulatory molecules. Following their migration to secondary lymphoid organs, the expression of these molecules together with the presentation of internalized antigens and secreted cytokines, will allow the DCs to regulate the magnitude and direction of T cell activation and thus the adaptive immune response. This response can manifest itself in one of two ways, either a B cell mediated humoral response, often directed towards extracellular pathogens or alternatively a $CD8^+$ cytotoxic T cell (CTL) induced cell mediated response to intracellular pathogens or transformed self cells.

Unlike the innate immune response, adaptive responses are reactions to specific antigenic challenges, which are made possible because each B or T lymphocyte expresses a unique antigen recognition receptor on its surface. When a specific lymphocyte recognizes its corresponding antigen this leads to its activation and subsequent clonal

expansion. These clones may then act as highly specific effectors, which can go on to clear the body of the original source of antigen. In this way the adaptive immune system generates a specific immune response towards a specific pathogen, while simultaneously ignoring self. As a means of generating the amount of diversity required for specific recognition of such a vast array of antigens, while safe guarding against self reactivity, lymphocytes must undergo an elaborate developmental process prior to their release into the periphery. For B cells this occurs within the bone marrow, while T cell precursors must first immigrate to the thymus to undertake their selection process.

1.2 Thymocyte Development

T cells arise from hematopoietic cells that migrate to the thymus (Figure 1). Their development does not occur cell autonomously but requires signals from non-hematopoietic cells in order to undergo maturation and learn to be restricted and tolerant to self through a process coined "central tolerance". Several explanations for the 'thymic dependency' of T cell development have been suggested, most of which agree that specialized stromal or epithelial cells in the context of the thymic microarchitecture provide unique signals that are required for proper thymocyte differentiation [1-3]. Additionally, these stromal cells reside in very distinct anatomic locations within the thymus, and the movement of precursor cells between these microenvironments is critical for the perception of differentiative signals [4, 5].

As outlined in Figure 1, T cells undergo a series of well-documented differentiation steps, which are typically defined based on the cell surface expression of

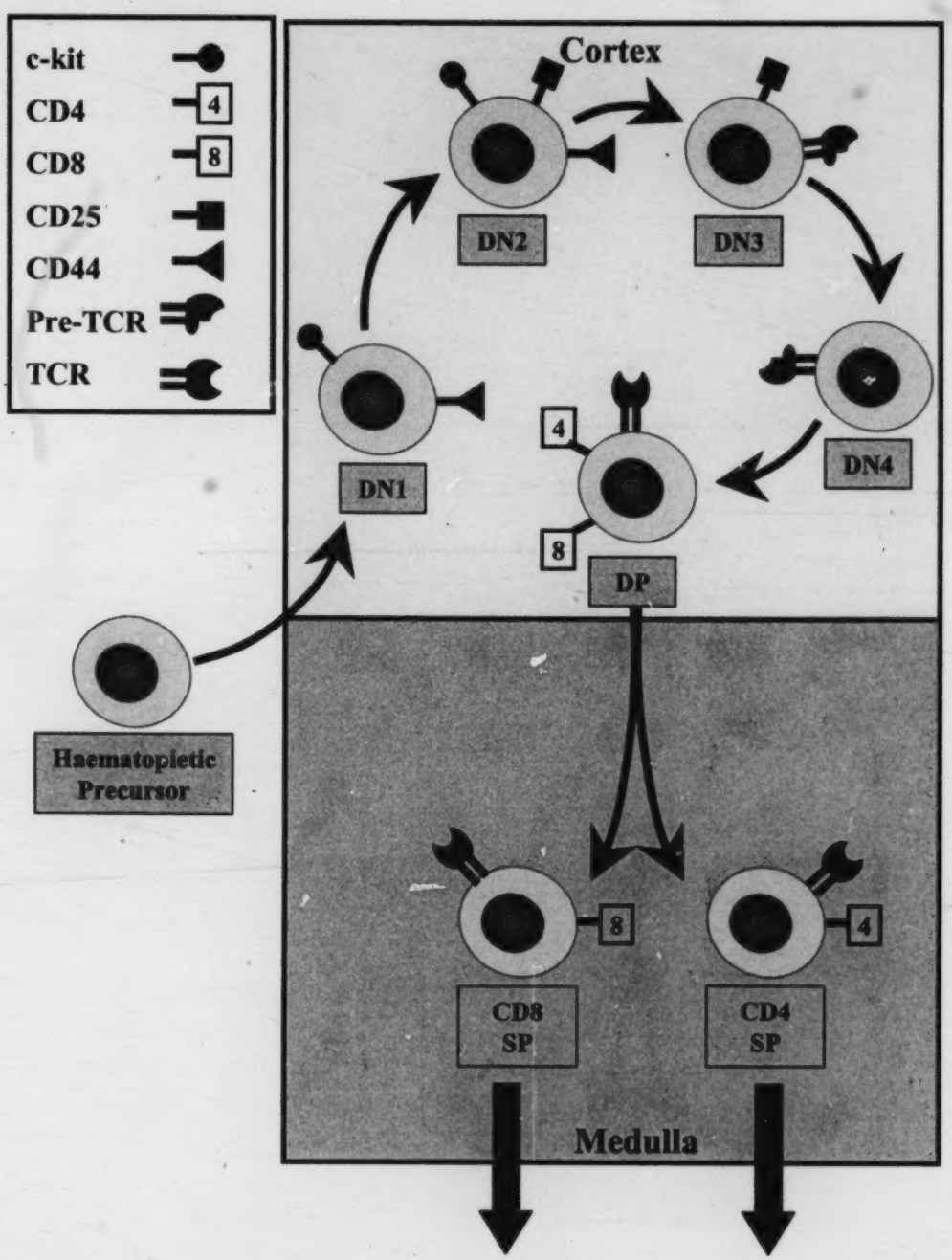


Figure 1: T cell development in the thymus

CD4 and CD8. Upon entry into the thymus at the cortical-medullary junction, precursors lack expression of CD4 and CD8 and are referred to as double negative (DN) [6]. Some groups also refer to this thymocyte subset as triple negative (TN), because they also lack expression of the CD3/T-cell receptor (TCR) complex [7]. These cells then migrate to the cortex where they progress through a number of stages, eventually up-regulating expression of both CD4 and CD8 molecules, marking their differentiation into double positive (DP) thymocytes. These DP cells have undergone productive rearrangement of the TCRβ chain and start to express a pre-TCR complex composed of TCRβ, a pre-Tα chain and the CD3 complex [8]. Expression of the pre-TCR then triggers rearrangement of the TCRα chain, expression of a mature CD3/TCR complex and allows these cells to participate in positive and negative selection. Those cells which are capable of only recognizing non-self antigens in the context of self-MHC down-regulate expression of either CD4 or CD8 thus becoming single positive (SP) thymocytes. At this point, the approximately 5% of thymocytes that pass selection find themselves within the medulla and are now capable of migrating from the thymus into the periphery as naïve T cells.

1.2.1 The Double Negative Stage

Further characterization of surface markers has revealed a more detailed stepwise developmental sequence within the DN stage, as this population can be further subdivided based on surface expression of CD44 [9], CD25[10] and c-kit[7]. These studies were able to define four DN thymocyte sub-populations labeled DN1-DN4, which occurred as follows: DN1, $CD44^+/CD25^-/c\text{-}kit^+ \rightarrow$DN2, $CD44^+/CD25^+/c\text{-}kit^+ \rightarrow$DN3, $CD44^-/CD25^+/c\text{-}kit^- \rightarrow$DN4, $CD44^-/CD25^-/c\text{-}kit^-$ (Figure 1) [11, 12]. It is at the DN3 stage that rearrangement of the TCR becomes evident. TCR gene rearrangement occurs

via V(D)J recombination, a process that is central to the development of the extensive diversity of both B and T cells [13]. Rearrangement of the TCRβ gene occurs first and its successful assembly and functional co-expression with CD3 and the invariant pre-TCRα chain is thought to trigger cellular proliferation, expression of CD4 and CD8 co-receptors and thus mark the transition from the DN to DP stages of development.

1.2.2 Pre-TCR Signaling and Thymocyte Development

Only thymocytes that have been able to successfully rearrange their TCRβ chains are allowed to progress from the DN→DP stage, this process is termed β-selection [14]. However, it is the co-expression of this TCRβ chain together with the pre-Tα and CD3 molecules in the pre-TCR that is thought to be important in triggering both survival and further differentiation of thymocytes, as cells that are unsuccessful in their rearrangement of the beta chain are thought to undergo apoptosis do to a lack of survival signals [15]. Current data also suggests that signaling though the pre-TCR may be ligand independent, and may instead be initiated through constitutive localization of pre-TCR complexes to membrane rafts [16]. Regardless of mechanism, signaling through the pre-TCR complex is responsible for initiation of numerous events leading to further thymocyte maturation including rescue from apoptosis, proliferation, downregulation of CD25, upregulation of both CD4 and CD8 coreceptors, cessation of TCRβ gene rearrangement (allelic exclusion), extinction of pre-Tα expression and initiation of mature TCRα chain rearrangement [17, 18]. In terms of signaling, although it cannot be said absolutely that the pre-TCR and mature αβTCR are coupled to identical sets of downstream pathways, current evidence from numerous studies in different mouse models looking at the signaling molecules Lck, Fyn [19], ZAP-70 [20], Syk [21], SLP-76 [22, 23] and LAT

[24] suggests that signaling is at least very similar [17]. Furthermore, direct analysis of pre-TCR signaling further downstream has demonstrated that, similar to the mature TCR, the pre-TCR can stimulate mitogen activated protein kinase (MAPK) signaling [25-27], Ca^{2+} release [28] and activation of transcription factors such as nuclear factor-κB (NF-κB) and nuclear factor of activated T cells (NFAT) [29, 30].

1.2.3 Positive and Negative Selection: The DP→ SP Transition

The fate of DP thymocytes and their transition to SP thymocytes is ultimately dictated by their TCR avidity for self-MHC ligands in the thymus. This step is of particular importance, as this is where positive and negative selection of thymocytes occurs; thereby shaping an individuals' functional T cell repertoire and ensuring that it is capable of recognizing antigens in the context of self-MHC while remaining unresponsive to self. A long-standing issue in thymocyte development is how signals through the same receptor, being the TCR, can generate such opposing outcomes as death and differentiation. Much data accumulated to date suggests that the outcome of TCR binding may differ depending on the extent to which it binds self-peptide-MHC on thymic stromal cells [4, 17]. This model proposes that extremely low avidity interactions result in 'non-selection' or death by neglect, low avidity interactions promote positive selection and high avidity binding triggers negative selection. However this raises another question as to whether strength of TCR binding manifests itself as either a qualitative or quantitative difference in terms of the signaling response.

Recent data suggests the existence of both qualitative and quantitative elements downstream of TCR engagement during selection of thymocytes. To begin, studies looking at TCR proximal signaling have shown that either augmentation or impairment of

these events can have profound effects on both positive and negative selection [31, 32]. In the most severe cases, impairment of TCR proximal signaling events can convert negative selection to positive selection, suggesting that these cell fates may lie on a signaling continuum [17]. The opposite can also be true as recent studies using activated forms of the GTPase Rac-1 in thymocytes resulted in a switch from positive to negative selection [33]. Interestingly, the alteration of more distal signaling machinery frequently affects either positive or negative selection, but often not both, suggesting qualitative differences may also exist between positive and negative selection further downstream. The best documented example suggests that positive selection occurs in response to low, sustained levels of extracellular regulated kinase (ERK) activation, whereas negative selection occurs following a large burst of ERK activation concomitant with jun-N-terminal kinase (JNK) and p38 activation. Several studies have shown that animals deficient for ERK display impaired positive but not negative selection, where as *grb*$^{-/+}$ animals which have defects in JNK and p38 activation have impaired negative selection, but positive selection is normal [20, 34-41]. Interestingly its also been reported that ERK has a lower threshold for activation than JNK or p38, perhaps explaining how quantitative differences in signal strength can induce qualitatively different outcomes [42]. When taken together, these studies suggest a model for thymocyte selection in which differences in TCR-ligand avidity dictate the strength of proximal TCR signals, which in turn determines the activation of a particular subset of downstream pathways. Interestingly, this model also allows for the possibility that other molecules such as co-receptors, co-stimulatory molecules and/or adhesion molecules could play an important

role in thymocyte selection, perhaps by directly amplifying the TCR induced signal or simply increasing the half-life of the TCR-peptide/MHC interactions [43].

The thymic stroma, on which this selection occurs, is complex and contains a number of cells important for thymocyte selection including epithelial cells and bone marrow derived cells such as macrophage and DCs. It has now become apparent that different stromal cell populations play very specialized roles in different stages of thymocyte differentiation, perhaps by providing appropriate accessory signals [3]. In terms of positive selection, although some debate still exists, the vast majority of experimental evidence suggests that thymic epithelial cells are the most efficient thymic stromal population capable of mediating this selective process. The first study to demonstrate this utilized MHC-mismatched bone marrow chimeras, and showed that non-bone marrow derived, radio-resistant thymic stromal cells were responsible for triggering maturation of immature thymocytes into mature SP T cells [44]. Many studies using a variety of model systems have confirmed this result and further demonstrated that not only are the epithelial cells required for positive selection, but that these cells are required to provide a sustained signal to DP thymocytes if they are to survive [3, 45-48]. For negative selection the majority of available data suggests that it is primarily bone marrow derived DCs and perhaps macrophage, that are important in carrying out central tolerance [49, 50]. Although it should be mentioned that some data also suggest a potential role for epithelial stromal cells in this selection process as well [51, 52].

1.2.4 Summary

Development of T cells occurs within the thymus and consists of a highly complex yet ordered series of events; the final outcome of which is a functional T cell repertoire

that although highly diverse in nature, is also both self-MHC-restricted and self-tolerant. From the time a T cell precursor arrives in the thymus, it must undergo numerous genetic alterations leading to expression of a unique TCR, endure a highly specific selection process through interactions with a variety of different thymic stromal cell populations; and if found suitable will only then receive the appropriate signals marking it for survival and export to the periphery as a naive T cell ready to respond to its specific antigen.

1.3 T cell activation

Due to the potent and potentially dangerous nature of T cell responses if activated under incorrect circumstances, T cell activation not only involves TCR binding its cognate MHC-peptide ligand, but a number of additional checkpoints and failsafe mechanisms have evolved to prevent aberrant T cell activation. For a naïve T cell to become fully activated *in vivo*, it must engage a professional antigen presenting cell (APC) (DC or perhaps macrophage or B cell) in both an antigen-specific manner through the TCR, and an antigen-independent manner through a variety of other receptors. These additional ligands include co-receptors, co-stimulatory molecules and adhesion molecules. In the absence of these molecules, not only will a T cell not be efficiently activated, but may become anergic (unresponsive) or even succumb to death.

1.3.1 TCR and Co-receptors

The trademark feature of all T cells is the expression of a somatically rearranged, clonal antigen receptor termed the TCR [53]. As previously discussed, every T cell is unique with regards to its TCR specificity, and it is for this reason that the overall T cell repertoire is extremely diverse. The TCR itself is a member of the Immunoglobulin (Ig)-

superfamily and is expressed as a heterodimer on the cell surface consisting of an α and β chain [54]. Although these chains directly recognize antigenic peptide/MHC, and thus dictate TCR specificity, neither chain demonstrates any direct signaling capacity. It is for this reason that the TCR is found associated with the invariant CD3 complex. The CD3 complex is composed of the transmembrane heterodimeric proteins CD3γ/ε, CD3δ/ε and the homodimer CD3ζ/ζ [55]. These proteins do not contribute to antigenic specificity, but instead contain one or more immunoreceptor tyrosine-based activation motifs (ITAM) required to transduce signals in response to TCR engagement.

Another set of surface molecules often found in close association with the TCR/CD3 complex is the CD4 or CD8 co-receptors. CD4, often associated with T-helper cells is found as a monomer and binds a non-polymorphic region of class II MHC; although some data suggests it may bind as a dimer [56, 57]. Alternatively, CD8 is always found as a dimer on the surface of CTL and recognizes an invariant region of class I MHC [58]. Its well established that the TCR binds with very low affinity to peptide-MHC complexes and so it is thought that simultaneous binding of CD4 or CD8 to the same MHC-peptide as the TCR may aid in stabilizing the TCR interaction [59, 60]. Additionally, the cytoplasmic domain of both CD4 and CD8 have been demonstrated to be non-covalently associated with the Src-family kinase (SFK) Lck (lymphocyte-specific cytoplasmic protein tyrosine kinase), which is crucial for TCR proximal signaling events [61]. Thus, binding of these co-receptors to MHC also acts to recruit Lck to the site of TCR engagement where it can more efficiently participate in TCR signaling.

1.3.2 Co-stimulation

Co-stimulatory signals have been defined as signals that synergize with or enhance signals provided when the TCR engages peptide/MHC, however cannot act autonomously [62]. The best characterized co-stimulatory molecule involved in naïve T cell activation is the Ig-superfamily member CD28 and its corresponding ligands B7-1 (CD86) and B7-2 (CD80). The role of CD28 is two-fold, first as a adhesion molecule which can act to help stabilize the interaction between the T cell and APC; and secondly its signaling provides an important second signal which promotes cytokine production and proliferation [63]. However in recent years it has become apparent that multiple other members of the Ig-superfamily (ICOS), as well as members of the tumor necrosis factor (TNF) family of surface receptors (4-1BB, CD27, OX40) can possess co-stimulatory activity [62, 64, 65]. Only a few of these are present constitutively on T cells (CD28 and CD27), whereas the rest are inducible, often appearing following TCR engagement. Constitutive expression indicates a very early involvement in naive T cell activation, whereas the inducible nature of many other co-stimulatory molecules suggests a role later in T cell activation; with it now appearing that many of these may be important in regulating the absolute number of effector T cells generated at the peak of the immune response and in dictating the frequency of memory T cells that subsequently develop [62, 64].

1.3.3 Adhesion molecules

T cells also express a variety of adhesion molecules including integrins and lectins, which play important role in mediating cell-cell and cell-extra cellular matrix (ECM) interactions; both of which are crucial for T cell development and activation. In

terms of activation, because the interaction between TCR/MHC-peptide is very low affinity, these adhesion molecules are important in the initiation and maintenance of T cell/APC contact. This initial transient binding of T cells to APCs is crucial in providing time for a T cell to survey the large numbers of MHC molecules being presented on each APC [66, 67].

Leukocyte function associated antigen-1 (LFA-1), which is found on the surface of all T cells, is one such member of the integrin family and its interaction with various intercellular adhesion molecules (ICAMs) on APCs is known to be crucial in mediating this initial binding. Like all members of the integrin family, LFA-1 is a heterodimeric protein consisting of a α and β chain and its importance in mediating T cell/APC interactions has been demonstrated in numerous studies showing that when blocked using antibodies, T cell responses are severely diminished [66, 68, 69]. Furthermore, although initial adhesion of LFA-1 to ICAM is relatively low-affinity, upon subsequent TCR recognition of its cognate MHC-peptide ligand a process termed inside-out signaling occurs resulting in a conformational switch to a high affinity state [70-72]. This increased adhesion of LFA-1 for ICAM aids in mediating prolonged T cell/APC contact and stabilizing TCR/co-receptor/co-stimulatory interactions allowing for enhanced T cell activation.

Lectins are another group of cell surface adhesion molecules, which often recognize carbohydrate motifs presented on glycosylated lipids and/or proteins through their conserved carbohydrate recognition domain [73]. To date only a few lectins have been directly shown to participate in mediating T cell/APC interactions, these are Dectin-1, DC-associated lectin-1 (DCAL-1) and Dendritic cell specific ICAM-grabbing non-

integrin (DC-SIGN), all of which are found on DCs [74-77]. DC-SIGN, which preferentially recognizes mannose has been demonstrated to mediate initial binding of T cells to DCs though a high affinity interaction with ICAM-3. Furthermore this interaction has even been suggested to direct recruitment of other adhesion molecules including LFA-1 to the APC-T cell synapse [74, 78]. Interestingly, unlike other adhesion molecules involved in T cell/APC adhesion, which do not discriminate between T cell binding to B cells, macrophage or DCs. The DC-SIGN/ICAM-3 interaction is specific for DCs, perhaps indicating some as of yet unidentified specialized role for DC-SIGN as an adhesion molecule.

1.3.4 Summary

Although the central receptor involved in T cell activation is the TCR, engagement of this receptor alone is insufficient for T cell activation. Instead T cell activation only occurs in response to the coordinated engagement of the TCR together with numerous antigen-independent receptors including co-receptors, co-stimulatory molecules and adhesion molecules, thus ensuring tight control of T cell responses.

1.4 T cell Signaling

Following two decades of scientific advances, a well defined model for TCR signal transduction is finally emerging (Figure 2). As previously discussed, the TCR itself contains no signaling motif, so following its recognition of a specific peptide/MHC complex signaling is initiated through the invariant CD3 chains. Each of these CD3 molecules contains one or more ITAMs, which upon TCR triggering become phosphorylated on two different tyrosine residues [79]. This phosphorylation is an

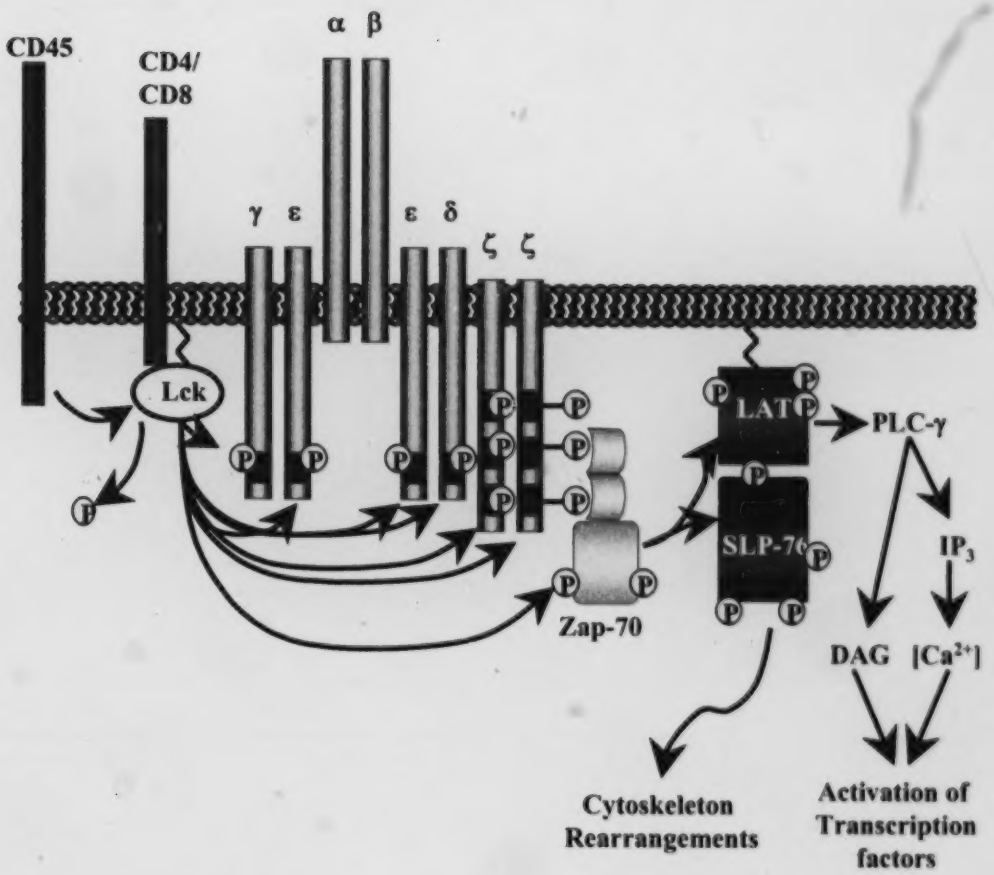


Figure 2: TCR proximal cell signaling

absolute requirement for any further signaling and is mediated by the Src-family of protein tyrosine kinases (PTKs). Lck is the predominant src-kinase involved in ITAM phosphorylation, while p59Fyn (Fyn) also has this capacity [80, 81]. The most important consequence of ITAM tyrosine phosphorylation is the recruitment of the Syk family kinase ZAP-70 (Zeta-chain associated protein-kinase 70kDa) via its tandem src-homology (SH)-2 domains [82, 83]. However, if recruitment of ZAP-70 was the only role for CD3, then there would be no reason to require the four different CD3 chains, as all are capable of binding ZAP-70 following phosphorylation. This lead to the differential signaling hypothesis, which is supported by a number of independent studies demonstrating that these chains, especially CD3ζ, are each capable of specifically recruiting a variety of different proteins [79]. In the case of zeta for example, it has been found to associate with many proteins involved in T cell activation and cytoskeletal rearrangements such as PI3K (phosphotidyl-inositol-3-kinase), Unc-119 (Uncoordinated-119), Shc, Vav and even actin itself [84-87].

ZAP-70 recruitment to the activated receptor complex results in its activation by a combination of Src-family mediated trans-phosphorylation and autophosphorylation [88, 89]. This catalytic activation of ZAP-70 enables it to subsequently phosphorylate the adaptor proteins linker for activated T cells (LAT) and SH2-domain containing leukocyte protein of 76kDa (SLP-76), which generates docking sites for numerous other downstream signaling proteins [90, 91]. This cascade leads to cytoskeletal rearrangements and hydrolysis of inositol-containing phospholipids, Ca^{2+} mobilization and activation of Ras/MAPK pathways, resulting in activation of the transcription factors NFAT, NFκB and AP-1 (activator protein-1); and ultimately culminating in specific gene

transcription leading to cellular proliferation, differentiation and acquisition of effector functions [55, 92, 93].

1.4.1 Lck

Lck is of critical importance in the initiation of TCR signaling and participates in multiple TCR proximal signaling events. Its importance has been illustrated using both Lck deficient cells lines in which TCR signaling is severely abrogated and $Lck^{-/-}$ mice which display a block in thymocyte development at the DN3 stage, with very few mature SP T cells being generated [80, 94]. These mice are deficient in signaling through the pre-TCR complex [95].

Similar to all SFKs, Lck contains SH2 and SH3 domains, a linker region and the kinase (SH1) domain. Two intramolecular interactions keep the enzyme in a closed "off" conformation: First is the binding of the SH2 domain to the phosphorylated COOH-terminal tyrosine residue (Y_{505}) and second this is further stabilized by binding of the SH3 domain to the linker region [96-98]. In addition, autophosphorylation of a second tyrosine residue (Y_{394}) within the catalytic site of the kinase domain is also required for full activity [99].

Severing of the interaction between the SH3 domain and the linker has been suggested to occur following binding of a higher affinity SH3 ligand [100]. Although CD2 and CD28 can potentially activate Lck through their SH3-binding motifs [101]; a newly identified protein, Unc-119 was found to be more potent in disrupting this interaction and furthermore through its association with CD3ζ, is more directly involved in signal generation through the TCR [87].

The other interaction is mediated by binding of the SH2 domain of Lck to the COOH terminal Y_{505}. This residue is maintained in a phosphorylated state in resting cells by C-terminal Src-family kinase (Csk) [102]. In resting cells Csk is found in proximity to Lck, however upon activation Csk is sequestered away from Lck shifting the balance in favor of dephosphorylation by the protein tyrosine phosphatase CD45 [103]. A role for CD45 in activation of Lck through removal of the negative regulatory phosphate was first established using CD45 deficient cell lines, where Lck was found to be hyperphosphorylated on this tyrosine (Y_{505}) resulting in impaired responses to antigen [104, 105]. However, the CD45-Lck relationship is not that straight forward, as studies have also demonstrated that CD45 is able to dephosphorylate the positive regulatory phosphate on Y_{394} within the activation loop within the kinase domain [106]. One possibility is that CD45 helps to keep Lck in a "primed" state, and that following TCR engagement during formation of the immune synapse, Lck is sequestered from CD45 allowing for autophosphoryation on Y_{374}. Although the mechanism of this regulation is not fully understood it has been proposed that it is due primarily to Lck inclusion in and CD45 exclusion from this early synapse [103, 107].

1.5 CD45

CD45 is a large, heavily glycosylated, transmembrane protein tyrosine phosphatase (PTPase) found on all nucleated cells of hematopoietic origin (Figure 3). It is also one of the most abundant cell surface glycoproteins, comprising up to 10% of total cell surface protein and therefore also contributing a significant fraction of cell surface carbohydrate [108]. CD45 ranges in size from 180kDa to 240kDa due to alternative splicing of three

exons corresponding to the most membrane distal region of the extracellular domain in addition to alterations in its glycosylation. This variability results in significant differences not only in the size, but also the shape and charge of this molecule. The cytoplasmic domain of CD45 is highly conserved and like many other receptor-like protein tyrosine phosphatases (RPTPs) it contains two PTPase homology domains designated D1 and D2. While D1 is the only catalytically active domain, D2 is thought to aid in stabilization of D1 and participate in substrate loading [109-111].

1.5.1 CD45 and T cell development

CD45-deficient mice generated through targeted ablation of exons 6, 9 or 12 have all demonstrated similar profound defects in thymocyte development, and impaired antigen receptor mediated signaling [112-114]. The progression of thymocytes from these mice through the different stages of development was analyzed by staining for CD4 and CD8. Comparison of the CD45 deficient mice to wild type mice reveals two significant blocks in development. The first of these was an approximately two-fold reduced number of DP thymocytes suggesting a minor block at the DN to DP stage of development. Further staining with CD44 and CD25 indicated this block was specifically occurring at the DN3 to DN4 stage of development [112, 114]. This is not surprising as progression beyond the DN3 stage requires signals through the pre-TCR which would not be expected to efficiently occur in the absence of CD45, as Lck would favor an inactive state [104]. Additionally there was an over five-fold decrease in the number of SP thymocytes suggesting the absence of CD45 also results in a severe block in the crucial DP to SP transition during which positive and negative selection is known to occur [112-114]. Furthermore, studies using fetal thymic organ cultures (FTOCs) [115]

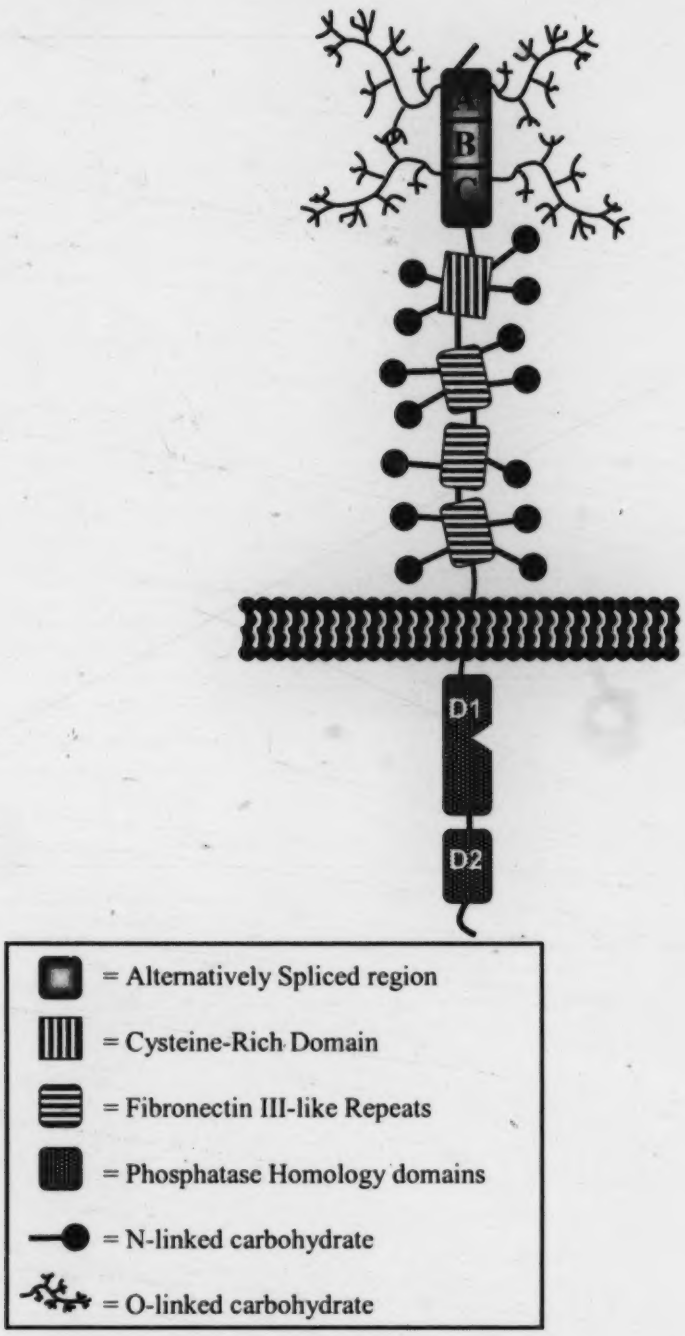


Figure 3: Schematic representation of CD45

and transgenic TCRs within the CD45 exon $12^{-/-}$ background [114] have also revealed an additional defect in positive and negative selection that appears to be related to the strength of antigenic stimuli. In other words, the signal threshold required to achieve both processes is increased when CD45 is not present. Further support that CD45 may be important for regulating signaling thresholds during T cell development came when it was observed that T cells that do survive in CD45 deficient mice have significantly increased autoreactivity [116]. Although these studies establish a role for CD45 in development, they fail to ascertain a role for the extracellular domain of this protein. Interestingly, studies looking at the surface expression of CD45 throughout development demonstrated regulated changes in CD45 isoform usage throughout thymocyte differentiation; therefore implying that specific isoforms of CD45, which differ only in their extracellular domain, may be important during specific stages of T cell development [117].

1.5.2 CD45 and Mature T cells

With regards to CD45 knock-out mice, these mice display greatly reduced numbers of mature peripheral T cells and as previously mentioned. Furthermore, those that are found often express TCR with high avidity for self-peptide/MHC, likely as a result of altered thymic selection processes [116]. In a study using the CD45 exon-6 ablated mice, those T cells that did make it to the periphery were tested for their ability to proliferate in response to both TCR cross-linking and PMA (Phorbol-12 Myristate Acetate) plus calcium ionophore stimulation, and were found to be deficient for both responses [113]. The involvement of CD45 in TCR signaling in response to antigen has also been shown using CD45 deficient cell lines, where CD45 was identified as an

obligate positive regulator of antigen signaling, as in its absence signaling in T cells is severely impaired [110, 118, 119].

1.5.3 The Extracellular Domain of CD45

Although the extracellular domain appears dispensable for reconstitution of antigen receptor signaling in several cell culture systems, it is subject to exquisite regulation [110, 120]. The extracellular domain is expressed as multiple iso- and glycoforms in a cell type, developmental stage and activation state dependant manner. Furthermore this pattern of expression and the overall structure of the extracellular domain are highly conserved across species suggesting functional importance *in vivo* [108]. Although it has been shown that different isoforms of CD45 do appear to have identical PTPase activity *in vitro*[108, 121], expression of different isoforms of CD45 in cell lines and mice deficient for this molecule indicated that some isoforms resulted in more efficient signaling and activation as compared to others [122-126]. Although many of these studies could not come to a consensus as to which isoforms resulted in most efficient signaling, the differences they observed suggest a role for the extracellular domain in regulation of the PTPase activity of CD45.

There are several hypotheses concerning how changes in the extracellular domain of CD45 can modulate the function of CD45. Based on studies utilizing chimeric EGFR-CD45 molecules which demonstrated that dimerization of CD45 may abolish its activity [127], spontaneous dimerization is one proposed model and suggests that an equilibrium of CD45 monomers to dimers exists on the cell surface, which differs based on alternative exon usage. Total CD45 phosphatase activity is predicted to be determined by this equilibrium [126, 128-130]. Alternatively, the hypothesis still favored by many is

that alterations in the extracellular domain of CD45, act to mediate interactions with specific ligands, although a CD45 specific ligand has yet to be identified [108, 131].

1.5.3.1 Isoform Expression

CD45 exists as multiple isoforms due to the alternative splicing of three exons encoding the membrane distal region of the extracellular domain. These exons 4,5 and 6 are designated A, B and C respectively, and can appear in any combination, resulting in the expression of up to 8 isoforms; of which the largest is CD45R(ABC) encoding all three and the smallest, CD45R(O) lacks all three. The expression of these isoforms is highly regulated and appears dependant on cell type, developmental stage and activation state [108]. Data accumulated from a number of different studies using a variety of techniques including fluorescent cell staining, histology, Northern blots and reverse transcriptase-polymerase chain reaction (RT-PCR) has resulted in some understanding of a general expression pattern for CD45 isoforms on developing and peripheral T cells. Thymic progenitors and a significant percentage of DN thymocytes appear to express one or more of the variable exons (primarily RA and RB) [117, 132-134]. Differentiation into DP thymocytes was found to trigger a switch to primary expression of the low molecular weight CD45R(O) isoform in addition to dramatically increasing the overall amount of CD45 on the cell surface [117, 132, 135]; this upregulation of overall CD45 expression remains high throughout the remaining life-span of a T cell. Similar to DN cells, SP thymocytes and naive peripheral T cells up-regulate expression of CD45 variants containing the variable exons (especially RB) [117]. Upon activation of mature peripheral T cells the CD45R(O) isoform once again becomes the most abundant CD45 species on the surface of the cell and this remains the case in memory T cell populations

[117]. Additionally, a study by Wallace *et al.* demonstrated a potential link between selection events and CD45 expression, as an increase in both CD45R(A) and R(B) was observed in thymocytes undergoing positive and negative selection. [136]. These data demonstrate that regulated changes in CD45 isoform expression throughout the life of a T cell do occur, which would suggest distinct roles for the specific forms of the extracellular domain; although the precise role of the extracellular domain and its complex regulation remain a mystery.

1.5.3.2 Glycosylation

The heavy glycosylation of CD45 is for the most part attributable to N-glycosylation throughout the extracellular domain, as well as O-glycosylation of the variable exons. The O-glycoconjugates consist primarily of core 1 and 2 oligosaccharides, with a proportion of the core 2 sugars being modified by sialic acid. Since this O-linked carbohydrate is found within the alternatively spliced regions, differential use of these exons can result in dramatic differences in the overall size, carbohydrate content, shape and charge of the extracellular domain [137, 138]; however glycosylation of CD45 not only depends on differential exon usage, but may also vary independently based on cell type, developmental stage and activation state of the cell [108]. The N-linked glycoconjugates found throughout the extracellular domain are mainly tetra- and triantennary complex-type sugar chains containing poly(N-acetyllactosamine) groups and exclusively α-2,6-linked sialic acid residues [138]. These N-linked carbohydrate additions have been demonstrated to be important for both cell-surface expression and stability of CD45 [139]. In addition to these sugar chains, a small amount of high-mannose type sugar chains were also detected [138]. Further study of the

trafficking of CD45 indicated that this high-mannose containing CD45 population exists as the result of an alternative trafficking pathway [140]. This study revealed that CD45 may be transported to the cell surface via two distinct routes: One conventional golgi-dependant pathway that allows fully processed CD45 to be expressed; and a second route that is independent of the golgi and allows expression of CD45 with immature carbohydrate (high-mannose).

1.5.3.3 Search for a Ligand

The existence of a ligand for CD45 is presumed based upon the observation that the overall features of the extracellular domain of CD45 are conserved across evolution and are similar to those of the receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) and platelet derived growth factor receptor (PDGFR) [108]. Interestingly, despite the 35% identity of the entire extracellular domain, the overall structure, regulation and way in which this protein is glycosylated remains extremely conserved, which has lead to the idea that proteins which do interact with CD45 may do so in a carbohydrate dependant manner. Following more than a decade of searching for such a ligand, a number of proteins have been observed to interact with the extracellular domain of CD45, all of which do so through its carbohydrates, although a specific ligand for CD45 has yet to be identified.

The first reported protein found to bind CD45 was CD22 [141, 142]. Found on B cells, CD22 is a member of the Siglec family, all of which possess a conserved membrane-distal immunoglobin domain that mediates binding to sialylated glycoproteins or glycolipids [143]. Although initially binding was believed to be specific for CD45RO, as an interaction between a soluble form of CD22 (CD22Rg) could be inhibited by the

UCHL-1 antibody (anti-CD45RO) [141]. Further study revealed that not only could CD22 bind multiple isoforms of CD45, but could also block calcium flux and phospholipase-γ phosphorylation in response to anti-CD3 [142]; a result that had been previously seen following co-ligation of CD3 and CD45 using antibodies [144]. However, it was finally determined that CD22 neither exclusively nor specifically binds CD45, but is a sialic acid binding lectin capable of binding N-linked α-2,6-linked sialic acids present on the surface of many glycoproteins [143, 145, 146]. Therefore, any of the effects CD22 binding had, could no longer be attributed to binding of CD45 alone, but could have instead been mediated through any number of different glycoproteins.

Galectin-1, is a member of the family of β-galactoside binding proteins, and is known to preferentially bind to clustered lactoamine residues on N- or O-linked glycans, including those on CD45 [147]. When initially discovered, it was thought that this CD45-galectin-1 interaction was important in the induction of apoptosis in immature thymocytes and activated T cells, perhaps through modulation of CD45 PTPase activity [147-149]. However, similar to CD22 binding, galectin-1 was subsequently shown to bind proteins other than CD45, including CD43 and CD7 [149, 150]. Although it is not currently fully understood how binding of each of these proteins to galectin-1 contributes to apoptosis of T cells; it is known that upon binding of galectin-1, spatial redistribution of these receptors is observed only on cells undergoing apoptosis, and that deletion of CD45, the CD45 cytoplasmic domain or CD7 abrogates galectin-1 mediated apoptosis [147, 149-151].

Another molecule found to interact with CD45 is Glucosidase II (GII), a protein important for carbohydrate processing within the endoplasmic reticulum, where it is

central to the protein folding process [152]. Similar to CD22 and galectin-1, this interaction also displayed lectin-like characteristics and moreover could be effectively inhibited in the presence of mannose [153]. Interestingly, this association appears developmentally regulated and may alter the carbohydrate content of CD45, potentially influencing thymocyte development via binding of CD45 on thymocytes to distinct lectins on stromal cells [154]. Supporting this notion, it was found that CD45 on immature thymocytes containing high mannose oligosaccharides has been observed to bind mannan binding protein (MBP), and the strength of this interaction may vary depending on GII activity [154, 155].

Multiple other studies have been done in an attempt to determine the specific ligand(s) for CD45, but these have proved unsuccessful. The external domain of CD45 was expressed as a soluble secreted glycoprotein, but this protein failed to specifically bind lymphoid cells or display any effect on lymphocyte adhesion, proliferation or cytolysis [156, 157].

1.5.3.4 Antibody Cross-linking of CD45

Numerous groups have taken advantage of the wide variety of monoclonal antibodies (mAb) to the CD45 external domain, to explore the outcomes of CD45 ligation and perhaps mimic the effects that binding of an endogenous ligand would have. These studies have looked at both soluble and immobilized antibody either alone or together with antibodies for other signaling molecules in order to dissect what role CD45 ligation may have in the regulation of these pathways. Although CD45 appears absolutely required for lymphocyte activation, numerous studies have indicated that co-immobilization of anti-CD45 with anti-TCR, anti-CD3 and/or anti-CD2, all of which

normally trigger activation, results in abrogation of these effects as indicated by decreased tyrosine phosphorylation, calcium flux, inositol phosphate activation and/or proliferation [158-162]. Interestingly, the observed effects appear dependant on the CD45 epitope bound, as these and other studies indicate that other anti-CD45 mAb have no effect or may even act to augment the response provided through CD3, CD2 or cytokines such as Interleukin (IL)-2 [158, 163, 164]. Regardless of the outcome, these effects seem to be mediated through the phosphatase domain as in many cases the addition of vanadate, a phosphatase inhibitor could abolish the anti-CD45 mediated effect. These results indicate that binding of CD45 antibodies to different epitopes might induce different intracellular signaling pathways. Even when immobilized alone, anti-CD45 has been shown to trigger significant cytoskeletal rearrangements, through the activation of Lck and other downstream signaling molecules such as paxillin and proline-rich tyrosine kinase 2 (Pyk2) [165, 166].

Interestingly, addition of some soluble anti-CD45 mAbs alone appeared to induce a different response, being either homotypic or heterotypic cell-cell adhesion, depending on the mAb used. These studies demonstrated that binding of specific anti-CD45 mAb could induce homotypic adhesion of thymocytes in an ICAM-3/LFA-1 dependant manner [167], homotypic adhesion of activated T cells in an LFA-1 dependant and independent fashion [168], or heterotypic adhesion between T cells and monocytes through an LFA-1/ICAM1 interaction [169]. When taken together, these studies provide evidence for differential regulation of CD45 dependant on epitope-specific antibody binding.

1.5.4 Summary

CD45 is a transmembrane protein known to be of critical importance within developing thymocytes and mature T cells for TCR proximal signaling events through its dephosphoryation of the Src-family kinases allowing for their subsequent activation. However, in contrast to its well characterized cytoplasmic domain and phosphatase activity, little is known regarding its large, complex and heavily regulated extracellular domain and what role it may play *in vivo*. However based on the evidence accumulated to date, one possibility is that altering of CD45 iso- and glycoforms may alter its specificity allowing it to bind different ligands, or even the same ligand in a different manner, resulting in participation in different intracellular signaling pathways.

1.6 C-type Lectin and Lectin like Receptors

C-type lectins are soluble or cell surface proteins characterized by their ability to recognize specific carbohydrates using a highly conserved carbohydrate recognition domain (CRD). On the basis of their molecular structure, two groups of membrane-bound C-type lectins can be distinguished, type I and type II which have their N-termini pointing outwards or into the cytoplasm, respectively [73]. The CRD itself contains a prototypic lectin fold, consisting of two anti-parallel β sheets and two α helices which form a calcium-binding pocket that is essential for carbohydrate ligand binding [73]. In many cases the requirement for calcium is absolute if stable binding to sugars is to occur and it is for this reason C (Calcium dependant)-type lectins got their name. Further efforts to define classical CRD containing lectins have resulted in two broad groups, those binding galactose-type carbohydrates, which contain the triplet QPD and those

binding mannose-type carbohydrates, which contain an EPN triplet [170]. Two such relatively uncharacterized lectins containing this EPN motif are Dectin-2 and CIRE. Both of these display a highly restricted expression profile, being found only in professional APCs, which suggests a potential role in mediating or modulating immune responses [171]. However, it is now apparent that many members of this family contain non-standard CRDs and likely do not coordinate calcium and so these proteins are said to have C-type lectin-like domains (CTLDs) and are considered lectin-like receptors. Furthermore, it is thought that many of these CTLDs may have non-carbohydrate ligands [73]. One prime example is Dectin-1 which although lacking some key residues involved in calcium coordination has been observed to stably bind T cells and perhaps act in some co-stimulatory capacity [171].

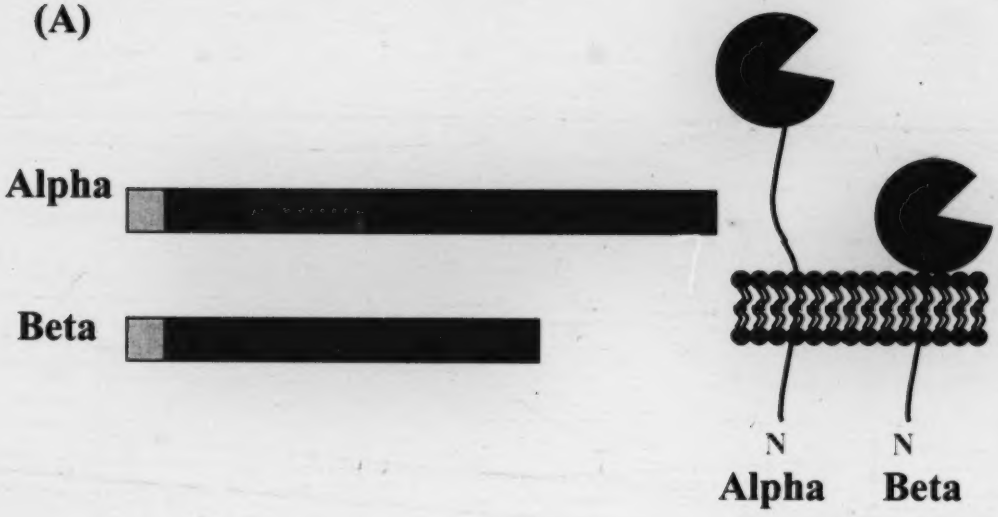
1.6.1 Dectin-1

Dectin-1 was originally identified through subtractive cloning of a mouse dendritic cell line against a macrophage line, with the intention of identifying genes uniquely expressed on DCs [76]. Dectin-1 is a type II C-type lectin like protein consisting of a short C-terminal cytoplasmic tail with a putative ITAM, a transmembrane region followed by a stalk and a single N-terminal CTLD. Although originally described as being unique to DCs, subsequent work demonstrated that macrophages, neutrophils and even some T cell lineages could express this lectin, and that in macrophages and DCs its expression was increased following activation [172, 173]. Given that its expression was for the most part restricted to APCs, Dectin-1 was found to be most abundant within lymphoid organs including the spleen, lymph nodes and thymus [76, 174]. Together with data indicating that the highest expression could be found within T cell areas of the

spleen and lymph nodes, as well as the medullary regions of the thymus, these observations suggest that Dectin-1 may play a role in T cell development and/or activation [175]. Further evidence for this came from three independent studies demonstrating that Dectin-1 could in fact bind both $CD4^+$ and $CD8^+$ T cells, with a preference for those that had been previously activated by Concanavalin A (ConA) [76]. Furthermore this binding enhanced T cell activation, when combined with sub-optimal concentrations of anti-CD3, as measured by an increase in proliferation, IFN-gamma production and up-regulation of activation markers including CD25, CD69 and CD40L [75, 76, 174]. These findings were subsequently confirmed in humans following the identification and isolation of the human dectin-1 homologue [176-178]. Studies in the human homologue also revealed the presence of a second truncated isoform of Dectin-1, termed Dectin-1β which lacked the extracellular stalk region as compared to the full length dectin-1α [176, 178]. This second isoform was subsequently shown to also exist in mice [178].

Apart from its role as a potential T cell co-stimulatory molecule, the best characterized aspect of this lectin is its role as a major β-glucan receptor [179]. It has been demonstrated that by binding β-1,3-linked and β-1,6-linked glucans it is able to efficiently recognize and trigger phagocytosis of a large variety of microorganisms, particularly fungi [75, 179, 180]. Furthermore, by signaling through its cytoplasmic ITAM motif via Syk kinase and in collaboration with TLR2 in macrophages, it can elicit a potent pro-inflammatory response that includes the release of cytokines such as TNF-α, IL-2 and IL-12, in addition to reactive oxygen species [181-184]. Interestingly, unlike many C-type lectins, Dectin-1 binds β-glucans in a calcium independent fashion, which

(A)



(B)

```
                                                                  3      3
            C   X       -CX X-       X  -    C       XX  X     X  X
Dectin-1 ---CLPNWIM HGKSCYLFSF SGNSWYGSKR HCSQLGAHLL KIDNSKEFEF
Dectin-2 ---CPNHWKS FGSSCYLIST KENFWSTSEQ NCVQMGAHLV VINTEAEQNF
CIRE     CRSCPWDWTH FQGSCYFFSV AQKSWNDSAT ACHNVGAQLV VIKSDEEQNF
SIGNR1   CRLCPWDWTF LLGNCYFFSK SQRNWNDAVT ACKEVKAQLV IINSDEEQTF

                                1    1                        2 21
         X              XXXX         X X   X  X         X    ***
Dectin-1 IESQTSSHRI NAFWIGLSRN QSEGPWFWED GSAFFPNSFQ VRNTVPQESL
Dectin-2 IT-QQLNESL -SYFLGLSDP QGNGKWQWID DTPFSQN-VR FWHPHEPN-L
CIRE     LQ-QTSKKRG -YTWMGLIDM SKESTWYWVD GSPLTLSFMK YWSKGEPNNL
SIGNR1   LQ-QTSKAKG -PTWMGLSDL KKEATWLWVD GSTLSSRFQK YWNRGEPNNI

           21                 22                  3
           CX X          X     C    X X C
Dectin-1 LHN-CVWIH- -GS--EVYNQ I.CNTSSYSI CEKEL----- -
Dectin-2 PEERCVSIVY WNPSKWGWND VFCDSKHNSI CEMKKIYL-- -
CIRE     GEEDCAEFR- -DD---GWND TKCTNKKFWI CKKLSTSCPS K
SIGNR1   GEEDCVEFA- -GD---GWND SKCELKKFWI CKKSATPCP- -
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Figure 4: C-type Lectins. (A) The two primary isoforms of Dectin-1, Dectin-2 and CIRE are the full length alpha isoform and the truncated beta isoform which lacks the stalk domain. (B) Comparison of the CRD region of Dectin-1, Dectin-2, CIRE and SIGNR1 with conserved hydrophobic core (**X**) and charged residues (**-**), Ca^{2+} coordinating residues (**1**-site 1; **2**-site 2; **3**-site 3), disulfide bonded cysteines (**C**), and the putative EPN mannose binding motif (*******) highlighted. Additionally, the WIH β-glucan binding site of Dectin-1 is underlined.

is not necessarily surprising as Dectin-1 lacks some potentially important calcium coordination residues within its CTLD (figure 4) [185, 186]. It is also interesting to note that β-glucans including laminarin and glucan phosphate, which potently block Dectin-1 binding to fungi, have no effect on binding to T cells [75]. This has lead to the suggestion that Dectin-1 may have two independent binding sites; one β-glucan binding site for innate immune recognition of pathogens and a second site of unknown specificity for binding an endogenous T cell ligand [179, 187].

1.6.2 Dectin-2

Originally described as Natural killer C-type lectin or NKCL (due to its close proximity to the NK gene complex) during a study using a mouse model of chronic myelogenous leukemia to identify genes over-expressed in the spleen [188]. Dectin-2, as it came to be called, was subsequently renamed following its identification using the same method as was used for Dectin-1 and is now known to belong to the dendritic cell immunoreceptor or DCIR family of lectins [189, 190]. Similar to Dectin-1, Dectin-2 also can be found as two major isoforms, an alpha form which contains a single N-terminal CRD, a stalk and transmembrane region; and a truncated beta form which lacks the stalk region. However, its cytoplasmic domain contains no identifiable motifs [189]. Dectin-2 also appears to be exclusively expressed in DCs and is found primarily in tissues such as the spleen, lymph node and thymus. Its expression is so restricted that some groups have even begun using its promoter as a means of DC-targeted gene expression to be potentially utilized in enhancing vaccination efficacy and regulating immune responses [191, 192].

Recent studies have now revealed that Dectin-2 may play a role in both the induction and maintenance of peripheral tolerance through its interaction with $CD4^+CD25^+$ T regulatory cells, although the precise mechanism by which Dectin-2 elicits its effects remain unknown [193]. Although no specific ligand is known for Dectin-2, this lectin does contain an EPN motif within its CRD suggesting a possible preference for mannose. Furthermore one report demonstrated that soluble Dectin-2 expressed from COS cells was specifically retarded on a mannose-sepharose column and could be eluted following Ca^{2+} chelation with EDTA [188]. A human homologue of Dectin-2 sharing 68% identity with its murine counterpart has also been identified, however little is known other than it too appears to be primarily expressed on dendritic cells [194].

1.6.3 CIRE

CIRE, also referred to as murine DC-SIGN (muDC-SIGN), was originally identified by two independent groups, both of which were looking for mouse homologues to the human DC-SIGN molecule [195, 196], however it now known that Sign-related gene 1 (SIGNR1) is the functional homologue to human DC-SIGN [197, 198]. CIRE is another type II C-type lectin with a single N-terminal CRD, stalk region, transmembrane section and a small cytoplasmic tail with no recognizable domains. It is found primarily in $CD11c^+$ $CD8\alpha^-$ myeloid derived dendritic cells within the spleen, and is down regulated upon their activation [195, 199]. Although nothing is currently known concerning CIRE ligands or function, it also contains a EPN motif within its CRD suggesting a potential preference for mannose.

1.6.4 Summary

C-type lectins are characterized by their ability to recognize specific carbohydrate moieties, and have been demonstrated to play many important roles within the immune system as innate pattern recognition receptors, during adhesion and homing or even in direct modulation of cellular activity. Dectin-1, Dectin-2 and CIRE are all known to be expressed on dendritic cells, which form the crucial link between the innate and adaptive immune responses. Moreover these lectins are found in the greatest abundance within lymphoid organs such as the thymus, where T cells develop, or within the spleen and lymph nodes where adaptive responses are first initiated; thus placing them in an excellent position from which to regulate T cell responses and development.

1.7 Overall Summary

CD45 is known to be absolutely required for proper T cell development and activation, however the precise role the extracellular domain plays in these processes is poorly understood. Studies using antibodies specific for CD45 have suggested that binding or cross-linking of particular portions of the extracellular domain of this protein can induce potent positive or negative signals, implicating the extracellular domain as being important in modulating T cell responses. Furthermore, a number of proteins have been identified that interact with CD45, all of which do so through the highly conserved carbohydrate moieties found throughout the extracellular domain of CD45; although a specific CD45 ligand has yet to be identified.

C-type lectins are a family of proteins characterized by their ability to recognize and stably bind specific carbohydrates. In recent years, many proteins belonging to this

family have been identified on professional APCs, and although initially thought to be primarily important in innate immune recognition of pathogens; it has now become apparent that these may serve many other roles including the initiation or regulation of the adaptive immune response through their binding of unidentified ligands on T lymphocytes.

1.7.1 Rationale and Hypothesis

The extracellular domain of CD45 only shows 35% homology between mammalian species, however the structure and carbohydrates presented on this molecule are highly conserved. Together with the finding that all proteins found to interact with CD45 do so in a carbohydrate dependant manner, we hypothesize that CD45 specific ligands will recognize specific oligosaccarides present on CD45 and furthermore that this binding may act to regulate T cells in the context of development and/or activation. Dectin-1, Dectin-2 and CIRE are all C-type lectins found primarily on DCs, however their precise roles have not been established. Dectin-1 has already been demonstrated to bind T cells, although its carbohydrate specificity and ligand remain unknown; and both Dectin-2 and CIRE display the EPN motif suggesting a preference for mannose, a carbohydrate found abundantly on the surface of some CD45 molecules. We propose that one or more of these lectins will aid in mediating T cell-APC interactions through CD45, and that binding of specific ligands such as these will be important for T cell development or activation.

CHAPTER II

MATERIALS AND METHODS

2.1 Cell lines

The murine T-lymphoma cell lines SAKR.TLS.12.1 (SAKR), BW5147 (BW), NZB and the CD45-negative variants (SAKR/T200$^{-/-}$ and BW/T200$^{-/-}$) were obtained from Dr. R. Hayman (The Salk Institute, La Jolla, CA), and maintained in DMEM (Life Technologies, Burlington, ON) supplemented with 8% defined calf serum (dCS) and 100μg/ml pen/strep. The murine T-lymphoma cell line YAC-wt and the CD45-negative variant (YACN1) were obtained from Jonathan Ashwell (NIH) and have been described previously [200], and were also grown in DMEM with 8% dCS. TP2, TEP and TZP are all CD8^{+ve} BW5147 cell lines that express a TAC, TAC/CD3 epsilon chimera and TAC/CD3 zeta chimera respectively. All of which were obtained from Lianjun Shen and were maintained in DMEM with 8% dCS.

The murine B lymphoma WEHI was obtained from Mike Gold (UBC) and has been previously described [201], these were grown in RPMI supplemented with 10% FCS, sodium pyruvate, L-glutamine and β-Mecaptoethanol (β-ME). Another murine B lymphoma A20.CY was obtained from American Type Culture Collection (ATCC) (Manassas, VA) and was maintained in DMEM supplemented with 8% dCS; this has also been previously described [202].

The dendritic cell line DC1.2 was obtained from Ken Rock and was maintained in DMEM supplemented with 8% dCS in tissue culture treated plates.

COS cells are derived from green monkey kidney cells and were provided by John Elliot (UofA). They were grown in DMEM supplemented with 8% dCS within tissue culture treated plates.

The CTL clone AB.1 (murine $H2^d$ anti-$H2^b$) which has been described previously [203], were grown in RPMI supplemented with 10% FCS, sodium pyruvate, non-essential amino acids, L-glutamine, pen/strep and β-2 mercaptoethanol. Cells were stimulated weekly with irradiated C57BL/6J spleen cells in media supplemented with IL-2 and experiments were performed 4-6 days following stimulation.

Thymic stromal cells (TSC) were isolated in our laboratory from thymi of C57B/6J mice according to the protocol by Niebergs, A.C. *et al.*[204]. Briefly, thymi from adult C57B/6J mice were removed. Connective tissue and other non-thymic tissue was removed using fine surgical instruments and then the thymi were cut into 1-2mm pieces. These pieces were placed into tissue culture dishes (Falcon) and cultured in RPMI supplemented with 15% FCS, L-glutamine, pen/strep, non-essential amino acids and sodium pyruvate. Under these conditions the adherent stromal cells migrate out of the disrupted thymic microenvironment and adhere to the tissue culture dish. These cells were maintained in culture.

2.2 Antibodies and Reagents

The pan-specific anti-CD45 mAb, I3/2.3 has been previously described [205]. The hybridomas producing the monoclonal antibodies MB23G2 and MB4B4 (anti-CD45RB), M1/8.9 and M1/9.8.1 (anti-CD45), M17/5.2 (anti-LFA-1), 145-2C11 (anti-CD3) were obtained through ATCC. PY72 (anti-phospho-tyrosine) was obtained from Dr. B. Sefton (Salk Institute, La Jolla, CA). F297 and H2 (anti-Glucosidase-GST) were

generated by Chris Arendt [206]. Hybridomas were grown in Protein free hybridoma medium-II (Life technologies, Burlington, ON) and the mAbs purified by ammonium sulfate precipitation followed by either protein A or protein G chromatography, if required. The polyclonal percentrin antibody was purchased from Berkeley Antibody Company (Richmond, CA). The fluorochrome-coupled mAbs RM4-4 (anti-CD4), 53.6.7 (anti-CD8), 1D3 (anti-CD19), 7D4 (anti-CD25), S7 (anti-CD43), IM7 (anti-CD44), MEL-14 (anti-CD62L), RA3-6B2 (anti-CD45 B220) and HI.2F3 (anti-CD69) were purchased from BD-Pharmingen (Gaithersburg, MD). Penta-His and AlexaFluor 647 conjugated penta-His were purchased from Qiagen (Mississauga, ON). HRP-, FITC- and PE-labeled secondary antibodies were purchased from Jackson labs. Streptavidin-HRP was purchased from Pierce (Rockford, IL).

Lipophilic membrane dyes, PKH67 (green) and PKH26 (red), carbohydrates (barley glucan, cellulose, dextran, dextran sulfate, dextrose, fructose, fructose-1-phosphate, fucose, fucoidin, galactose, galactose-6-phosphate, galactose-6-sulfate, glucosamine, glucosamine-2,6-disulfate, glucose, glucose-6-phosphate, glucose-6-sulfate, heperan sulfate, α-lactose, β-lactose, laminarin, maltose, mannan, mannose, mannose-1-phosphate, mannose-6-phosphate, N-acetyl galactosamine, N-acetyl glucosamine, N-acetyl mannosamine, pullulan, sucrose and xylose) were purchased from Sigma Chemicals (St. Louis, MO). Additional carbohydrates (Galactose-α1,3-galactose, galactose-4-sulfate, heparin and sulfatides) were purchased from Calbiochem (La Jolla, CA).

Cytochalasin E, used at 10μM to disrupt actin filaments and sodium vanadate which blocks phosphatases at 250μM were purchased from Sigma (St. Louis, MO). PP2,

a Src-family kinase inhibitor used at 10μM, PTPase CD45 inhibitor used at 3.5μM, Piceatannol a p72Syk inhibitor used at 10μM and bisindolylmaleimide 1 (Bim1) a PKC inhibitor used at 2μg/ml were all purchased from CalBioChem (La Jolla, CA). Herbimycin A is a protein-tyrosine kinase inhibitor used at 10μg/ml was purchased from GibcoBRL (Gaithersburg, MD).

FITC labeled zymosan, a yeast derived particle was purchased from Molecular Probes (Eugene, OR).

Sulfo-SBED is a chemical cross-linker and was purchased from Pierce Biotechnology (Rockford, IL).

2.3 RNA isolation and cDNA generation

For isolation of RNA from cell lines, approximately $1x10^7$ cells were washed 3X with PBS and resuspended in 1 ml of Trizol reagent (GibcoBRL, Gaithersburg, MD). Alternatively, RNA could also be directly isolated from spleen or thymic tissue by homogenization of 100mg or less of tissue in 1 ml Trizol. Samples were incubated for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes. 0.2ml of chloroform was then added, the mixture was thoroughly vortexed and incubated for 2 minutes at room temperature. Samples were then centrifuged for 15 minutes at 12000xg at 4°C. The clear upper aqueous phase was then carefully isolated, and the RNA precipitated by addition of 0.5ml isopropyl alcohol. Samples were then incubated for 10 minutes at room temperature followed by centrifugation at 12000xg for 10 minutes at 4°C. The pellet was then washed with 70% ethanol by centrifugation at 7500xg for 5 minutes at 4°C. The RNA pellet was then briefly air dried (5-10 minutes) and resuspended in 100μl or RNAse free H_2O.

The isolated RNA was then used for the generation of cDNA using the superscript first strand synthesis RT-PCR kit (Invitrogen, Burlington, Ontario). Approximately 5μg of RNA was added to 0.5μg oligo dT and 1mM dNTP in a 10μl reaction which was incubated at 65°C for 10 minutes and subsequently placed on ice for 1 minute. 2μl of 10xbuffer, 4μl of 25mM $MgCl_2$, 2μl of 0.1M DTT and 1μl of RNAse out were then added. This mixture was heated at 42°C for 2 minutes and 50units of superscript II-reverse transcriptase was added. This reaction was then incubated for 50 minutes at 42°C. The reaction was then terminated by heating to 70°C for 15 minutes and followed by addition of 1μl of RNAse H and incubation at 37°C for 15 minutes. At this point the cDNA is ready for use in polymerase chain reaction (PCR).

2.4 Cloning of lectins

Dectin-1 and Dectin-2 were first amplified by polymerase chain reaction (PCR) from cDNA generated from the DC1.2 dendritic cell line. This was accomplished using the forward primer DecI-4a (5'-GGTTTGGCTTAGTGAGCCTCATCC-3') and reverse primer DecI-4b (5'-CTCACATACATTTACAGTTCCTTCTCACAG-3') for Dectin-1 and the forward primer DecII-2a (5'-ACCCCTGACCTTCTGAACATACAC-3') and the reverse primer DecII-2b (5'-CGGCCTCTGTCAGATGTACAGGCC-3'). The reactions contained 1μg of cDNA, 5μl 10x HotstarTaq buffer (Qiagen, Mississauga, Ontario), 3μl of 25mM $MgCl_2$, 1μl 10mM dNTPs, 1μl of 10mM forward/reverse primers, 1μl HotstarTaq polymerase (Qiagen) and 36μl H_2O. The reactions were then incubated at 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 62°C for 30 seconds and 1 minute at 74°C. The reactions then underwent a final extension step for 10 minutes at 74°C, followed by incubation at 4°C. The resulting PCR products were run on a 0.9%

agarose gel, visualized using ethidium bromide and a UV transilluminator and the 700 (Dectin-1β) and 833 (Dectin-1α) or 773 (Dectin-2α) and 674 (Dectin-2β) base pair products removed and purified using the QIAquick gel extraction kit (Qiagen, Mississauga, ON). 4μl (80ng) of the purified Dectin-1 or Dectin-2 insert was then ligated into the pDrive PCR cloning vector (Qiagen, Mississauga, ON) by incubation together with 50ng of pDrive and the provided ligation buffer in a 10μl reaction at 4°C for 30 minutes. Following the ligation, the plasmid was then transformed into the Qiagen EZ competent cells. 2μl of ligation mix was added per tube of cells and incubated on ice for 5 minutes. This was followed by a brief heat shock step at 42°C for 30 seconds, followed by another incubation on ice for 2 minutes. 250μl of fresh LB was then added to each mixture and incubated at 37°C for 25 minutes. 150μl of each mixture was then directly plated on LB plates supplemented with 100μg/ml ampicillin, 50μM IPTG, 80μg/ml X-Gal and allowed to incubate at 37°C overnight. A minimum of five white colonies were then selected and used to inoculate 5ml of fresh LB supplemented with 100μg/ml ampicillin, which were then grown overnight at 37°C and 250rpm. These cultures were then used to create glycerol stocks to be stored at -80°C in addition to being used to generate purified plasmid using QIAprep spin mini-prep kit (Qiagen, Mississauga, ON). These plasmids were initially screened by restriction digest with BamHI and XbaI, with correct inserts generating a fragment of approximately 850bp (Dectin-1α), 725bp (Dectin-1β), 780bp (Dectin-2α), 685bp (Dectin-2β). If correct, plasmids were then sent for sequencing at the Molecular Biology Services Unit (Biological Sciences, University of Alberta) using the primers DecI-4a and DecI-4b for Dectin-1 and DecII-2a and DecII-2b for Dectin-2 in order to verify the isolation of a correct, mutation free lectin insert.

Once correct clones were generated in the pDrive vector, these were subsequently subcloned into the pcDNA3 vector. The Dectin-1 insert was removed by digestion with XbaI and BamHI, whereas the Dectin-2 insert was isolated by digestion with KpnI and XbaI and both were purified using QIAprep spin mini-prep kit (Qiagen, Mississauga, ON). Purified pcDNA plasmid was also prepared following XbaI and BamHI or KpnI digestion followed by dephosphorylation using bacterial alkaline phosphatase (Invitrogen, Burlington, Ontario). Insert and plasmid were then ligated using T4 ligase at 15°C for 3 hours, and subsequently transformed into DH5α *E. coli* by heat-shock. Colonies resulting from overnight growth on LB (ampicillin) plates were then used to inoculate 5ml cultures which were subsequently used to generate glycerol stocks and also used for isolation of plasmid using the QIAprep spin mini-prep kit (Qiagen, Mississauga, ON). Purified plasmids were then screened by restriction digest with XbaI and BamHI or KpnI. Endo-free plasmid preparations (Qiagen, Mississauga, ON) were then performed on successful clones to obtain DNA to be used in transfection of eukaryotic cells.

CIRE was originally amplified from cDNA prepared from whole C57B/6J spleen tissue, using the primers mDCSIGN-1a (5'-CGGAATTCCTGAGAAGTGG CTGTGAAACATGAGTG-3') and mDCSIGN-1b (5'-GCTCTAGAGCATGGTGGA GGGAGTTGGCCATCACTT-3'), under conditions identical to those used for amplification of Dectin-1 and -2. The CIRE insert was then gel purified as discussed for Dectin-1 and -2, however following gel purification the insert was digested with EcoRI and XbaI and purified using phenol:chloroform extraction/ethanol precipitation. Using T4 ligase, the insert was then directly ligated into purified pcDNA3 that had been previously digested with EcoRI and XbaI. The ligation was then transformed into DH5α

E. coli by heat-shock. Colonies resulting from overnight growth on LB (ampicillin) plates were then used to inoculate 5ml cultures which were subsequently used to generate glycerol stocks and also used for isolation of plasmid using the QIAprep spin mini-prep kit (Qiagen, Mississauga, ON). Purified plasmids were then screened by restriction digest with XbaI and EcoRI. Endo-free plasmid preparations (Qiagen, Mississauga, ON) were then performed on successful clones to obtain DNA to be used in transfection of eukaryotic cells.

For cloning of Dectin-1, Dectin-2 and CIRE into the N-terminal 6xHis-tagged vector pQE30, the extracellular portions of these lectins were amplified by PCR (conditions as above) using the relevant pcDNA3-lectin clones as template. Primers used were as follows: for Dectin-1 Hisfor-dec1 (5'-CGCGGATCCGCGAATTCAGGGAGAA ATCGACAC-3') and Hisrev-dec1 (5'-TCCCCCGGGGGACATTTACAGTTCCTTCTC ACA-3'); for Dectin-2 Hisfor-dec2 (5'-CGCGGATCCGCGGACCAGCCCAGTAGAAG ACTA-3') and Hisrev-dec2 (5'- TCCCCCGGGGGACAGGCACTCATAGGTAAATCT-3'); and for CIRE Hisfor-DCSIGN (5'-CGCGGATCCGCGCCCAGTTCTCAGGAAGA AAAC-3') and Hisrev-DCSIGN (5'-TCCCCCGGGGGAATGGTGGAGGGAGTTGGC CAT-3'). In addition, the Hisfor primers all contained BamHI cut site, and the Hisrev primers contained SmaI cut sites. All inserts were then gel purified and digested with SmaI and BamHI. The pQE30 vector was also prepared by digestion with BamHI and SmaI restriction enzymes, followed by gel purification using the QIAquick gel purification kit (Qiagen). The insert was then ligated into pQE30 using T4 ligase for 3 hours at 15°C. This ligation mix was then used to transform the M15 strain of *E. coli* which also contains the pREP4 plasmid confering kanamycin resistance and also

constitutively expresses the *lac* repressor [207]. Transformants were plated on LB plates supplemented with 100μg/ml ampicillin and kanamycin 25μg/ml. Colonies were used to inoculate 5ml of LB media, which was subsequently used to make glycerol stocks and for plasmid purification using the QIAprep mini-prep spin kit (Qiagen, Mississauga, ON). Plasmids were initially screened by digestion with SmaI and BamHI and plasmids recovered from positive colonies were subsequently sent for sequencing to the Molecular Biology Services Unit (Biological Sciences, University of Alberta) using the corresponding Hisfor and Hisrev primers. Positive clones were then screened for recombinant protein expression using the Ni-NTA spin kit (Qiagen, Mississauga, ON).

2.5 Transfection and generation of stable clones in COS and YACN1 cell lines.

Transfection of COS cells with lectin/pcDNA3 constructs was accomplished using the Effectene transfection reagent, a non-liposomal lipid formulation (Qiagen, Mississauga, ON). The day before transfection, $5x10^5$ COS cells were seeded in a 60mm tissue culture treated plate and grown overnight in DMEM/8% dCS at 37°C. 1μg of endotoxin-free prepared plasmid was used for transfection. Media was changed 6 hours following procedure and cells were allowed to recover overnight under normal growth conditions. Selection of successful transfectants was then performed by passaging cells 1:10 into DMEM supplemented with 8%dCS and 2mg/ml G418 (GibcoBRL, Gaithersburg, MD). Following the recovery of an antibiotic resistant population, single cell clones were then isolated by serial dilution and then grown out and screened by RT-PCR for expression.

Transfection of YACN1 T cells was done by electroporation using the Gene Pulser (Biorad, Hercules, CA). To begin, $6x10^6$ cells were washed 3x in PBS and

resuspended in 800µl PBS. 10µg of lectin/pcDNA3 construct was then added and the mixture was placed on ice for 30 minutes followed by transfer into a 0.4cm cuvette (BioRad, Hercules, CA). Cells were then electroporated once at 300V and allowed to recover for 48 hours in DMEM/8%dCS. Selection and isolation of clones was then performed as described above.

2.6 Thymocyte/Splenocyte Isolation and ConA Blast Generation.

Thymi and spleens were removed from C57BL/6J mice and placed in DMEM with 8% dCS and subsequently homogenized. The resulting suspension was subjected to NH_4Cl lysis to remove erythrocytes. Thymocytes and splenocytes were washed 2X with PBS and quantified using a hemocytometer, and resuspended at between 10-50x10^6/ml to be used in subsequent experiments.

In order to generate ConA blasts, splenocytes were resuspended at 10x10^6/ml in RMPI supplemented with 10%FCS, Sodium pyruvate, L-glutamine, pen/sprep and β-ME. Concavalin A was then added to 2µg/ml, and cells were incubated in 25cm^2 flasks (Corning, Corning, NY) at 37°C for 48 hours prior to use.

2.7 AB.1 Stimulation

AB.1 cells were stimulated on immobilized anti-CD3 with or without lectin. Anti-CD3 (145-2C11) was first immobilized in a ninety-six well flat bottom Falcon 3912 microtitre plate (Becton Dickinson, Oxnard, CA) between 0.001µg/ml to 10µg/ml for 1 hour at 37°C. In situations where lectin was co-immobilized, the appropriate lectin was added to 15µg/ml and the plate was incubated overnight at 4°C. Plates were then washed 2X with PBS and blocked with 2% bovine serum albumin (BSA) in PBS for 60 minutes

at 37°C. This was followed by two washes in PBS before the addition of a set number of AB.1 cells, depending on the experiment to be carried out.

2.8 Conjugate Assay

Conjugate assay were performed in one of two ways, either using cell staining in a FACS based assay, or adhesion of cells onto an established monolayer in a plate based assay.

2.8.1 Plate-Based Binding Assay

In the plate based conjugate assay, control COS cells and those expressing the lectins were grown to approximately 80% confluence in a 6 well tissue culture treated plate. The COS cell monolayer was then gently washed once with pre-warmed PBS. Target T-cell lines or previously isolated *ex vivo* populations were washed 2X in PBS and resuspended at $2x10^6$ cells/ml in PBS/2%dCS. 3 mL of target cells were added per well and the plate incubated for 45 minutes at 37°C. Unbound cells were then removed and the plate gently washed 2X for 1 minute with pre-warmed PBS, after which fresh PBS was added and cells were visualized by light microscopy. Four representative fields of view were analyzed by counting COS cells and bound T cells and determining the ratio.

Alternatively target T cells were stained using the PKH67 green lipophilic dye (Sigma Chemicals). These cells were washed 3X in 4%dCS/PBS and incubated at 37°C for a 1 hour rest period. These cells were then be used as described above and bound cells visualized by fluorescent microscopy.

2.8.2 Fluorescent Activated Cell Sorting (FACS) Adhesion Assay

Control or lectin-expressing YACN1 cells and target T cells were washed with PBS and stained with green PKH67 and red PKH26 lipophilic dyes, respectively (Sigma

Chemicals, St. Louis, MO). The cells were then washed 3X in 4% dCS/PBS, and incubated at 37°C for a 1 hour rest period to prevent dye transfer. Cells were resuspended at $1x10^6$ cells/ml (YACN1) and $2x10^6$ cells/ml (target T cells) in 4% dCS/PBS. Equal volumes (100µl) of the cells were mixed and spun down at 700rpm for 2 minutes and subsequently co-cultured for specific times at 37°C. Following this incubation period, cells were quickly vortexed and fixed by addition of 200µl of 4% paraformaldehyde. Conjugate formation was measured by flow cytometric analysis; conjugates were defined as pairs of cells fluorescing both red and green. The percent conjugation was measured by dividing the number of YACN1 conjugates/total number of YACN1.

2.9 Recombinant lectin Expression and Purification

In order to express and purify the 6xHis-lectins, 50ml of LB culture media containing both ampicillin (100µg/ml) and kanamycin (25µg/ml) was inoculated with appropriate M15-his-dec1, M15-his-dec2 or M15-his-CIRE and cultures, and grown overnight at 37°C and 250rpm. One litre of pre-warmed media containing (100µg/ml) ampicillin was inoculated with 50ml of the overnight culture and grown for approximately 1 hour until OD_{600} equaled 0.6. At this point IPTG was added to a final concentration of 1mM to induce expression, and cultures were incubated for an additional 4 hours at 37°C. Cells were then harvested by centrifugation at 4000xg for 20 minutes at 4°C. Cells were lysed by resuspension in TE (10mM Tris, 1mM EDTA) with 1mg/ml lysozyme at 3ml per gram wet weight and overnight incubation at 4°C, followed by sonication (6X 30 seconds). Inclusion bodies containing recombinant lectins were spun down at 22000xg for 25 minutes and washed twice with TE by centrifugation at 22000xg

for 20 minutes. Pellets were then lysed overnight in lysis buffer (6M GuHCl, 10mM Tris, 100mM NaH_2PO_4, pH 8.0). Following incubation, the entire lysate was then loaded onto a freshly regenerated Nickel-NTA agarose column containing 2mL packed beads (Qiagen, Mississauga, ON). Columns were washed twice with 10mL wash buffer (8M Urea, 10mM Tris, 100mM NaH_2PO_4, pH 6.0). The recombinant lectins were eluted in 10mL of elution buffer (6M Urea, 10mM Tris, 100mM NaH_2PO_4, pH 4.5). Following this, eluates were dialyzed against refolding buffer (550mM Arginine, 264mM NaCl, 11mM KCl, 50mM Tris, 5mM $CaCl_2$, 5mM $MgCl_2$, 10mM reduced Glutathione, 1mM oxidized Glutathione, pH8.2) supplemented with 6M Urea. This was followed by dialysis into refolding buffer alone and then extensive dialysis into TBS supplemented with 2mM $MgCl_2$ and 2mM $CaCl_2$ or 1x PBS. At this point the lectins were quantified using a standard BCA assay and purity established by running the lectins on a 12% SDS-PAGE gel, followed by Coomassie staining.

Refolding of Dectin-1 was verified by binding to zymosan. 15μg/ml His-Dectin-1 was added to excess quantities of zymosan for 2 hours on ice, following which the quantity of soluble versus zymosan bound (refolded) Dectin-1 was quantified by SDS-PAGE. FACS was also performed to detect zymosan bound His-Dectin-1 using anti-His AlexaFluor 647 antibodies.

2.10 Purification of Immune Cell Populations

Magnetic activated cell sorting (MACS) was used for the enrichment of three different cell populations: dendritic cells, T cell and B cells. The Pan T cell isolation kit (Miltenyi Biotec, Auburn, CA) utilized negative selection by removing all non-T cells from a heterogenous population. Total splenocytes were first obtained from the spleen of

one C57B/6J mouse. Following 2X washes in PBS, these were resuspended at 10^7 cells/40µl in buffer A (PBS supplemented with 0.5% BSA and 2mM EDTA). 10µl of Biotin-Antibody cocktail (specific to CD11b, B220, DX5 and Ter-119) was added and left for 15 minutes on ice. 30µl of buffer A was then added together with 20µl of anti-biotin microbeads per 10^7 cells and left for 20 minutes on ice. Cells were then washed 2X in buffer A and resuspended in 500µl buffer A per 10^8 cells/500µl (minimum 500µl). A MACS LS column was applied to the magnet and pre-rinsed with 3ml of buffer A following which the resuspended cells were then added. The effluent containing unlabeled cells was collected as this represents the enriched T cell fraction, to be used in subsequent experiments.

B cells were purified using a positive selection kit (Miltenyi Biotec) specific for CD45R (B220). Heterogeneous cell populations were first collected from spleens of C57B/6J mice, washed 2X with PBS and resuspended at 10^7 cells/90µl in buffer A. 10µl of CD45R(B220) microbeads were then added per 10^7 cells, mixed and left on ice for 20 minutes. Cells were washed 2X with buffer A and resuspended at 10^8 cells/500µl (minimum 500µl). A MACS LS column was applied to the magnet and pre-rinsed with 3ml of buffer A, following which the resuspended cells were then added. The column was washed 3X with 3ml of buffer A, removed from the magnet and 5ml of buffer A was added. The bound fraction representing enriched B cells was then immediately flushed out using the provided plunger.

DCs were also purified using a positive selection kit (milenyi Biotec), specific for CD11c. Thymi were isolated from C57B/6J mice as previously discussed. Thymi were then minced and resuspended in digestion buffer containing 1xPBS, 1.2U/ml dispase

(Sigma, St. Louis, MO), 0.1% DNase (Sigma, St. Louis, MO), 1.6ml collagenase (Sigma, St. Louis, MO) and 2% dCS and incubated at 37°C for 35 minutes with rotation. Following three washes with PBS, cells were resuspended at 10^8 cells per 400µl. 100µl of anti-CD11c microbeads were then added per 10^8 cells and left for 20 minutes on ice. The remainder of the purification protocol is as described for B cells.

2.11 Soluble Lectin Binding Studies

Binding of lectins to various T cell populations was evaluated using a FACS based assay. Cells to be stained were first washed 3X in PBS, quantified using a hemacytometer and suspended at $10x10^6$ cells/ml in PBS. $1x10^6$ cells were then incubated on ice for 30 minutes with 15µg/ml His-tagged lectin (saturating quantity), followed by 2X washes with PBS to remove excess lectin. Cells were then resuspended in 100µl PBS/2%dCS and incubated for 30 minutes on ice with 1:50 dilution of AlexaFluor 647 conjugated anti-penta-His antibody (Qiagen, Mississauga, ON). In situations where co-staining for cellular markers was performed, flourochrome-coupled antibodies for CD4, CD8, CD19, CD25, CD44, CD62L and/or CD69 were also added at 1:50 (FITC-coupled) or 1:100 (PE-coupled) dilutions. This was followed by three washes in PBS and cells were then fixed by addition of 300µl of 4% paraformaldehyde. Staining was assessed using flow cytometric analysis. Intensity of staining obtained through this method was compared to background fluorescence obtained when cells were stained with no antibody or AlexaFluor 647 anti-penta His antibody alone.

2.12 Cellular Spreading Experiments

To study cellular spreading induced following treatment with various antibodies and/or lectins, antibodies and lectins were first immobilized on a 96 well plate. In

experiments looking at anti-CD45 induced spreading, I3/2, MB23G2, MB4B4. M1/9 and M1/89 were all immobilized at 15μg/ml at 4°C overnight. Alternatively, when studying lectin induced spreading, 145-2C11 (anti-CD3) was immobilized at 10, 0.1 or 0.01μg/ml either alone or together with 15μg/ml lectin overnight at 4°C. Plates were washed 2X with PBS and blocked for 60 minutes at 37°C with 2%BSA/PBS. Following this, plates were again washed 2X with PBS. The relevant T cells (AB.1, YAC or *ex vivo* T cells) were then washed 2X with PBS and counted with a hemacytometer. Cells were resuspended in PBS/2%dCS at $1x10^6$cells/ml and 100μl of cells were added to each well. Cells were then incubated for 45 minutes at 37°C, unless otherwise stated. Spreading was then visualized by light microscopy and quantified by calculating percentage of cells spread as compared to total cells within a representative field of view. All experiments were repeated at least 3X and in some circumstances pictures were taken.

2.13 Degranulation Assay

Degranulation, as measured by the release of serine esterase, was assayed as previously described [208]. AB.1 cells were washed 3X by centrifugation in PBS and resuspended in 2% dCS in RPMI. AB.1 were then stimulated by addition of $2x10^5$ cells/well in 150μl directly to wells of 96 well plates previously coated with 145-2Cll (anti-CD3 antibody) with or without 15μg/ml lectin. Plates were then incubated at 37°C for 5 hours after which 25μl supernatant was assayed for Benzyloxy carbonyl-L-lysine Thiobenzyl (BLT)-esterase activity [208], developed for 30 minutes and read in a kinetic microplate reader (Molecular Devices, Sunnyvale, CA) at 405nm. All conditions were performed in quadruplicate and the standard deviation calculated.

2.14 Interferon (IFN)-γ ELISA

IFN-γ release was measured using a sandwich ELISA. AB.1 cells were washed 3X by centrifugation in PBS and resuspended in 2%dCS in RPMI. AB.1 cells were then stimulated by addition of $1.5x10^5$ cells/well in 150µl directly to wells of 96 well plates previously coated with 145-2Cll (anti-CD3 antibody) with or without 15µg/ml lectin. Plates were then incubated at 37°C for 24hours after which 100µl supernatant removed, diluted (1:50, 1:200 and 1:400) and added to a 96 well plate previously coated in IFN-γ capture antibody (BD Pharmingen, Mississauga, ON). Plates were then sealed and incubated for 2 hours at room temperature, followed by 5X washes in PBS/0.1% Tween-20. 100µl of working detector containing biotinylated detection antibody and Avidin-HRP reagent (BD Pharmingen, Mississauga, ON) was then added to the plate and incubated at room temperature for an additional hour. Following another 10X washes with PBS/ 0.1% Tween-20, 100µl of substrate solution was added and the plates were allowed to develop for 30 minutes at room temperature at which time 50µl of 2N H_2SO_4 was added to stop the reaction. Plates were then read in a kinetic microplate reader (Molecular Devices, Sunnyvale, CA) at 450nm. All experiments were performed in quadruplicate and the standard deviation calculated.

2.15 Sodium Chlorate Block

In order to block cell surface sulfation of proteoglycans we grew cells in either sulfate free Fischer medium (GibcoBRL, Gaithersburg, MD) supplemented with 8% dialyzed dCS and 50mM sodium chlorate or low sulfate Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham Base (Sigma, St. Louis, MO) supplemented with L-glutamine, L-lysine, L-leucine, L-methionine, $CaCl_2$, $MgCl_2$, sodium bicarbonate, 8% dialyzed dCS and 50mM sodium chlorate, which competitively inhibits formation of 3'-

phosphoadenosine-5'-phosphosulfate (PAPS), the high energy intermediate for cell sulfation reactions. Cells were incubated for 48hours at 37°C, prior to use.

2.16 Chemical Cross-linking

The chemical cross-linker to be used, Sulfo-SBED (Pierce, Rockford, IL), was first attached to the bait (lectin). Sulfo-SBED was resuspended 10μg/μl in DMSO and 1μl was added to 250μl of lectin (200μl/ml), thereby giving a four fold molar excess of sulfo-SBED. The reaction was then carried out at room temperature for 30 minutes in the dark. The reaction was then dialyzed against 500ml of transfer buffer (50mM HEPES, 150mM NaCl) overnight at 4°C in the dark to remove non-reacted cross-linker. Following dialysis, SBED-labeled lectin was added at 50μg/ml to $30x10^6$ YACN1 T cells and allowed to incubate for 45 minutes on ice in the dark. Cells were washed 2X in PBS and UV induced cross-linking was performed for 5 minutes with 5x15watt lamps at a distance of 5cm (Stratalinker 2400). Cells were then lysed in 1ml of 1% NP40 and the nuclei were spun down at 12000rpm for 10 minutes. 50μl was mixed with an equal volume of 2X Laemmli reducing sample buffer (RSB) and lysates separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Proteins were transferred to Immobilon P (Millipore Corporation, Bedford, MA) and immuno-blotted using strepavidin-HRP, as Sulpho-SBED deposits a biotin residue unto target upon mixing with reducing sample buffer (RSB).

Strepavidin-agarose beads were added to the remaining supernatant and incubated overnight at 4°C on a rotator. Beads were then washed 3X in PBS/1% NP-40 and 2X in PBS. Bound proteins were eluted using 1x RSB and boiling for 5 minutes, and were run on a 12% SDS-PAGE gel. Eluted proteins were then detected by silver staining, the gel

was first placed in 45% EtOH/30%acetic acid for 20 minutes, followed by 2X washes for 20 minutes in 30% ETOH and 3x10 minute washes in ddH_2O. The gel was then stained using 0.1%$AgNO_3$ for 20 minutes followed by a quick rinse in ddH_2O. Developing was done in 100ml of 2.5% Na_2CO_3+250µl formaldehyde until protein bands were evident, at which point the reaction was stopped by extensive rinsing with H_2O.

2.17 Clustering Experiment

In these experiments T cells (AB.1, BW, YAC or *ex vivo* T cells) were first washed 2x in PBS and quantified using a hemocytometer. Cells were then resuspended in PBS/4%dCS at $1x10^6$cells/ml. In cases where inhibitors were used, cells were pretreated for 15 minutes on ice with 10µM Cyto E (Sigma, St. Louis, MO), 250µM vanadate (Sigma, St. Louis, MO), 10µM PP2 (CalBioChem, La Jolla, CA), 3.5µM PTPase CD45 inhibitor (CalBioChem, La Jolla, CA), 10µg/ml Herbimycin A, 10µM Picatannol (CalBioChem, La Jolla, CA) or 2µg/ml Bim1 (CalBioChem, La Jolla, CA); likewise anti-LFA-1 antibody was used at 20µg/ml for blocking, cells were pretreated for 15 minutes on ice. Following this, the appropriate anti-CD45 antibody was added (I3/2, MB23G2, MB4B4, M1/9 or M1/89) at 15µg/ml or anti-CD45 Fab fragment (I3/2Fab or 23G2 Fab) at 10µg/ml and cells were incubated at 37°C for 1 hour unless otherwise stated. Following this incubation, cells were visualized using light microscopy and clustering was graded on a 5 point scale (0→no clustering, 1→under 25% of cell clustered, 2→ 25-50% cells clustered, 3→ most cells clustered, 4→all cells in small clusters and 5→ all cells in large clusters), pictures were also taken.

2.18 Generation of Antibody Fab fragments

Papain was pre-activated with 10mM Cysteine in 1.25mM EDTA at pH7.0 for 15 mintutes at 37°C. 5-10mg of antibody was then added at a 1:50 (w/w) ratio of enzyme to antibody, and allowed to incubate at 37°C for 2 hours. Cleavage was stopped by addition of iodiacetamide to 25mM. Digested Fab fragments were then dialyzed into PBS.

2.19 SDS-PAGE and Immunoblot

Following activation of cells with soluble or immobilized antibodies and/or lectins, the cells were directly lysed by addition of an equal volume of 2X Laemmli reducing sample buffer. Lysates were subsequently separated on 8.5% or 12% SDS-PAGE gels. Proteins were then transferred to Immobilon P (Millipore Corporation, Bedford, MA) and immunoblotted using various primary antibodies. Secondary antibodies coupled to horseradish peroxidase (anti-mouse ^{HRP}, anti-rat^{HRP} or protein-A^{HRP}) were used to visualize the proteins by an enhanced chemilluminescence (ECC) system (NEN Life Science Products, Boston, MA).

2.20 Statistics

Where error bars are included, they represent standard deviation calculated based on four independent experiments unless otherwise indicated. All statistical analysis was performed using Microsoft Excel.

CHAPTER III

LECTIN CHARACTERIZATION

3.1 Introduction

Dectin-1, Dectin-2 and CIRE all belong to the C-type lectin family and are primarily expressed on DCs or other professional antigen presenting cells within primary and secondary lymphoid organs. This restricted expression on professional APCs may suggest a role for these lectins in mediating interactions with T cells and thus modulating the adaptive immune response. In fact, there is already evidence to suggest that Dectin-1 binds T cells and may possess some co-stimulatory activity, although the specific ligand and mode of action for this remain unknown [76, 174]. Such a role for Dectin-2 and CIRE has not been established.

The focus of this chapter is to establish a role, if any, for these lectins in binding and perhaps modulating the activity of different T cell populations both in the context of T cell development and activation. In particular, I was interested in whether any potential lectin-T cell interactions may be mediated through binding to CD45, an abundant and highly glycosylated PTPase; and if so, how this binding may modulate CD45 activity, as previous work with CD45 indicated that binding of antibodies or lectins such as galectin-1 to the extracellular domain may have consequences in terms of its activity [148, 165, 168, 169]. Interestingly both Dectin-2 and CIRE are known to contain the conserved EPN motif suggesting a potential preference of mannose-based ligands, of which mannose is known to be a major carbohydrate component present on some species of CD45 expressed on thymocytes.

3.2 Dectin-1, Dectin-2 and CIRE are expressed on thymic stroma

In order to test whether Dectin-1, Dectin-2 and CIRE played any role in T cell development and which isoforms are relevant, it was first important to confirm that these lectins are expressed within the thymus; and if so, then establish which isoforms are present. Due to the lack of specific antibody reagents, I utilized an RT-PCR based approach to amplify any relevant isoforms first from whole C57B/6J mouse thymus. As all three lectins were found to be expressed within the thymus (Figure 5B), I then used MACS to enrich dendritic cell and non-dendritic stromal cell populations in order to determine whether these lectins are expressed strictly in DCs or if they are perhaps found on other stromal populations (Figure 5A). Although Dectin-1 appears specific to DCs, Dectin-2 and CIRE could both be found in non-DC stromal populations in significant amounts (Figure 5C). Additionally, RT-PCR on a thymic epithelial cell line also revealed expression of Dectin-2 and CIRE, indicating that this non-DC expressing population of stromal cells could be epithelial cells (Figure 5D). With regards to isoform usage, it appears that both isoforms of Dectin-2 were detected, and only one isoform of Dectin-1 and CIRE were present, corresponding to Dectin-1β and CIREα (Figure 5).

3.3 Dectin-1 and CIRE mediate adhesion to T cells in the conjugate assay

In terms of establishing a role for these lectins in modulating T cell responses, it was first important to assess whether they could stably adhere to T cells; for Dectin-1 this had already been observed [76]. To assess adhesion, both the alpha- and beta-isoforms of each lectin were expressed on adherent COS cells (Dectin-1 and Dectin-2) or the CD45-deficient T lymphoma cell line called YACN1 (CIRE), and analyzed for their ability to mediate or increase adhesion to various T cell populations (Figure 6A

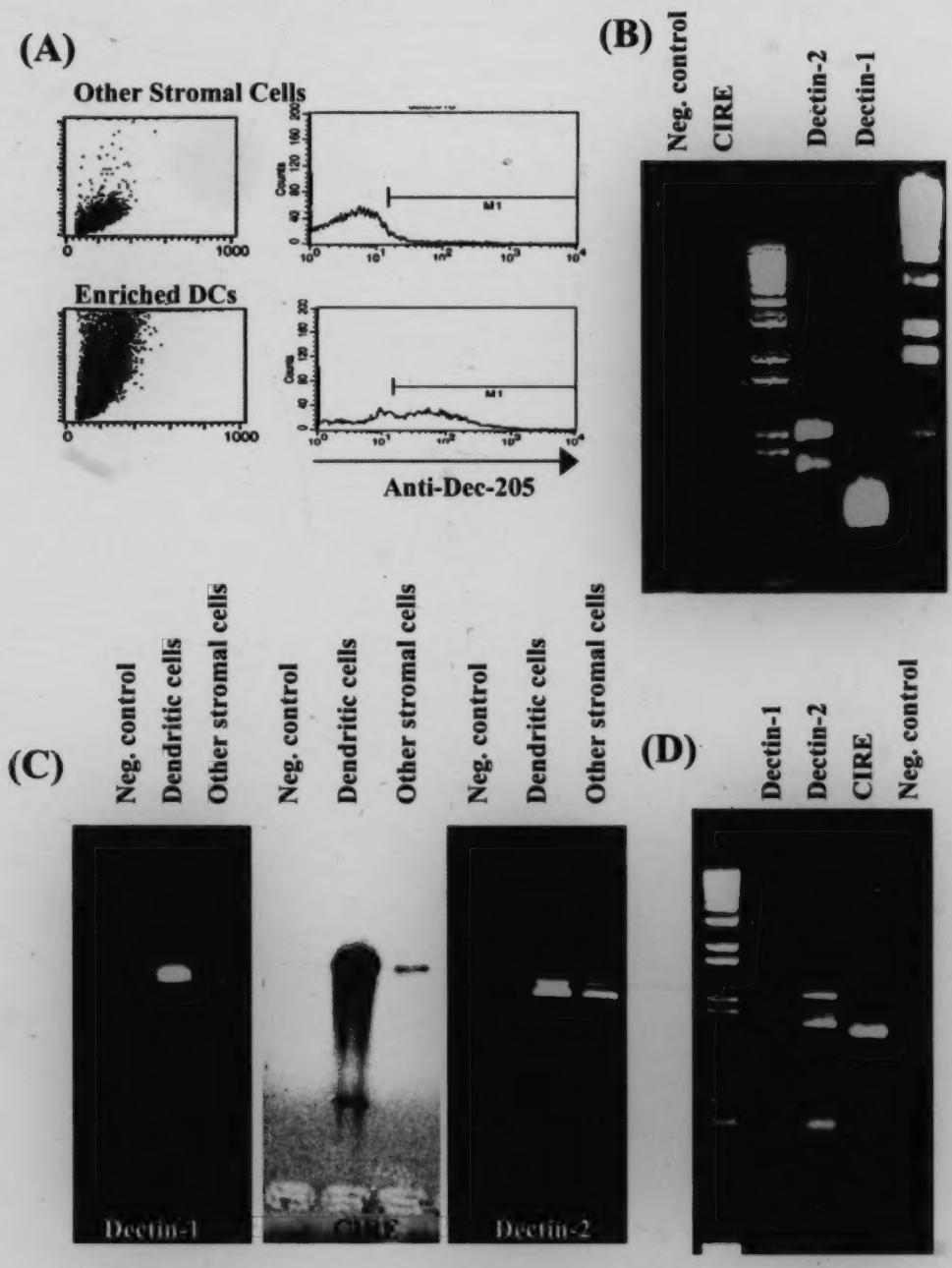


Figure 5: Dectin-1, Dectin-2 and CIRE are expressed on thymic stromal populations. Whole thymi were isolated from C57B/6J mice and the dendritic cells enriched/removed by MACS for CD11c. (A) DC enriched population stained for DC marker Dec205. RT-PCR was then performed from whole thymus (B), enriched thymic DCs, remaining stromal cells (C) and cultured Thymic epithelial cells (D).

and 7A). Using a plate bound assay where lectin expressing or control COS cells were first grown as a monolayer, and fluorescently labeled T cells subsequently added and observed for stable binding to COS cells. I observed that expression of both isoforms of Dectin-1 could significantly enhance COS cell binding to thymocytes, in addition to mature and activated T cells (Figure 6B, 6C, 6D and 6E). In contrast, Dectin-2 appeared to demonstrate no significant binding to any of the studied T cell populations (Figure 6C, 6D and 6E). For studying CIRE, a FACS based approach was used where lectin-expressing or control YACN1 cells and target T cells were stained red and green respectively with lipophilic dyes, and the formation of hetero-conjugates determined by the appearance of a double stained population. Using this assay, I determined that although CIRE was unable to bind thymocytes, both isoforms were capable of stably binding mature and activated T cells (Figure 7B, 7C and 7D). It is also of interest to note that YACN1 cells are themselves T cells and when made to express either isoform of CIRE this resulted in a drastic increase in clustering of these cells in culture, further suggesting some role for this lectin in binding to T cells (Figure 7E). Together this data argues against any role for Dectin-2 in T cell activation or development, but does suggest a role for both Dectin-1 and CIRE in modulating T cell responses and for Dectin-1 alone in T cell development. In addition, given these results I was unable to distinguish any significant difference in binding between the different lectin isoforms.

3.4 CIRE and Dectin-1 bind T cell lines, independent of CD45

In order to directly demonstrate binding of these lectins to T cells, I generated soluble forms of each of these lectins by fusing a 6xHistidine Tag to the N-terminal end of the extracellular domain and expressed the proteins in *E. coli* (Figure 8A). To confirm

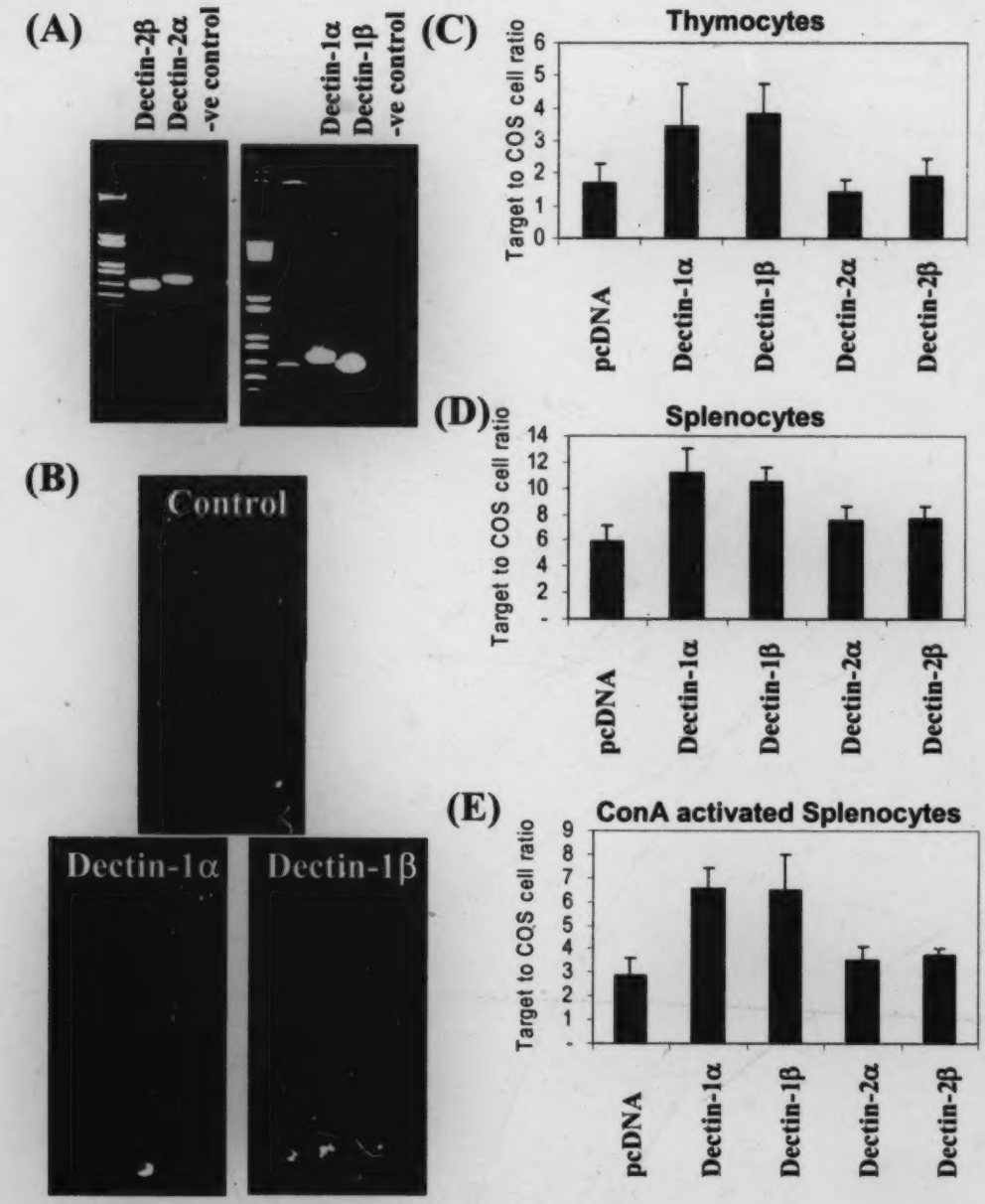


Figure 6: Dectin-1, but not Dectin-2 bind T cells and thymocytes. Dectin-1 and Dectin-2 were expressed in COS cells as confirmed by RT-PCR (A). Control and lectin expressing cells were grown as a monolayer onto which various T cell populations were added and analyzed for binding, as described. Representative Data for Dectin-1 binding to thymocytes is shown in (B). Adhesion is presented graphically to Thymocytes (C), Splenocytes (D) and ConA activated splenocytes (E) as a ratio of bound T cells to COS cells. This data represents an average of three independent experiments.

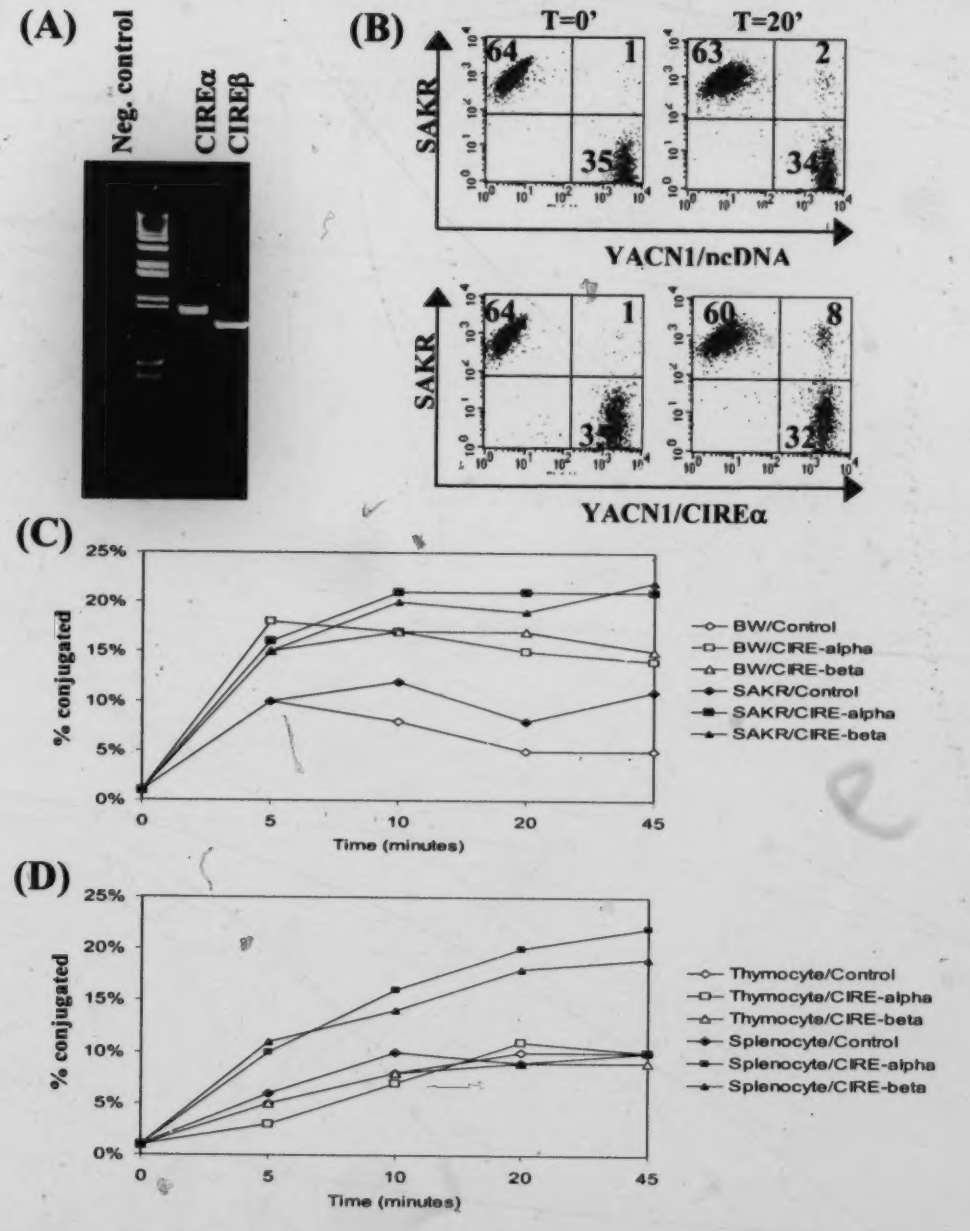


Figure 7: CIRE binds mature T cells, but not thymocytes. CIRE was expressed in YACN1 cells as confirmed by RT-PCR (A). Control and CIRE expressing cells were then used in a FACS based conjugate assay, as described. Representative data of SAKR adhesion to CIREα expressing YACN1 following 0 and 20 minutes is shown in (B). Adherence to cell lines SAKR and BW (C) and *ex vivo* thymocytes and splenocytes (D) is presented graphically over time as percent YACN1 conjugated=UR quadrant/ (UR+UL quadrants)x100%. This figure represents the average of two independent experiments.

(E)

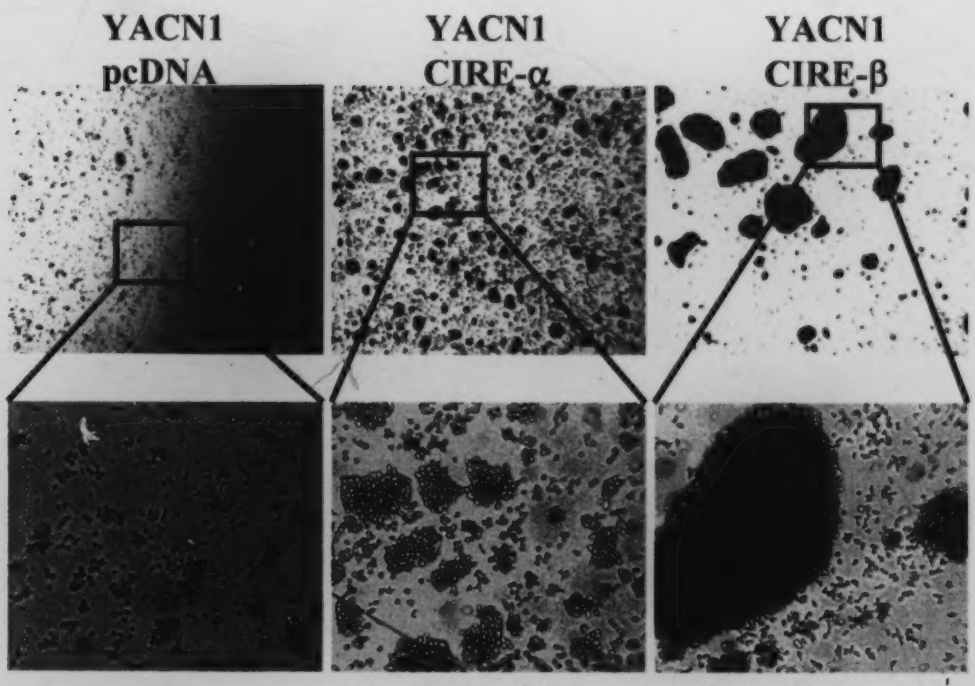


Figure 7: (E) YACN1 cells expressing CIRE-α, CIRE-β or control plasmid were grown in culture overnight and pictures taken 12 hours later.

binding of these lectins to T cells and potentially establish a role for CD45 in the interaction, I studied binding using saturating quantities of both of these lectins to CD45-positive and negative T cell lines including BW, YAC and SAKR (Figure 8B and 8C). To do this, soluble lectins were first added to cells in PBS and subsequently detected using a directly coupled anti-His antibody and FACS analysis. Dectin-1 demonstrated strong binding to all the evaluated cell lines and clones, and binding often appeared enhanced in the absence of CD45, suggesting that Dectin-1 can bind independently of this transmembrane PTPase (Figure 8B and 8C). Likewise, CIRE also bound most of the cell lines tested, but binding was significantly lower than that observed for Dectin-1. CIRE also displayed enhanced binding to CD45 negative cell lines, suggesting that CIRE could also bind independently of CD45 (Figure 8C). This data confirms that both lectins do in fact bind T cells and although both CIRE and Dectin-1 do appear to bind independently of CD45, it does not necessarily indicate that they cannot recognize CD45 when present.

3.5 CIRE and Dectin-1 bind multiple *ex vivo* T cell populations

Due to the irregular nature of many transformed T cell lines, it was also important to determine whether CIRE and Dectin-1 could directly recognize *ex vivo* thymocytes and T cells. Splenic T cells and thymocytes were harvested from freshly isolated spleen and thymic tissue from C57B/6J mice. Lectin binding was analyzed in a similar fashion to that described for the T cell lines, however co-staining was also performed by addition of directly coupled antibodies for CD4 and CD8 (Figure 9A). Dectin-1 strongly bound both thymocytes and splenocytes. In terms of thymocytes, Dectin-1 appears to have a preference for early DN and DP populations in addition to CD4 SP thymocytes, while

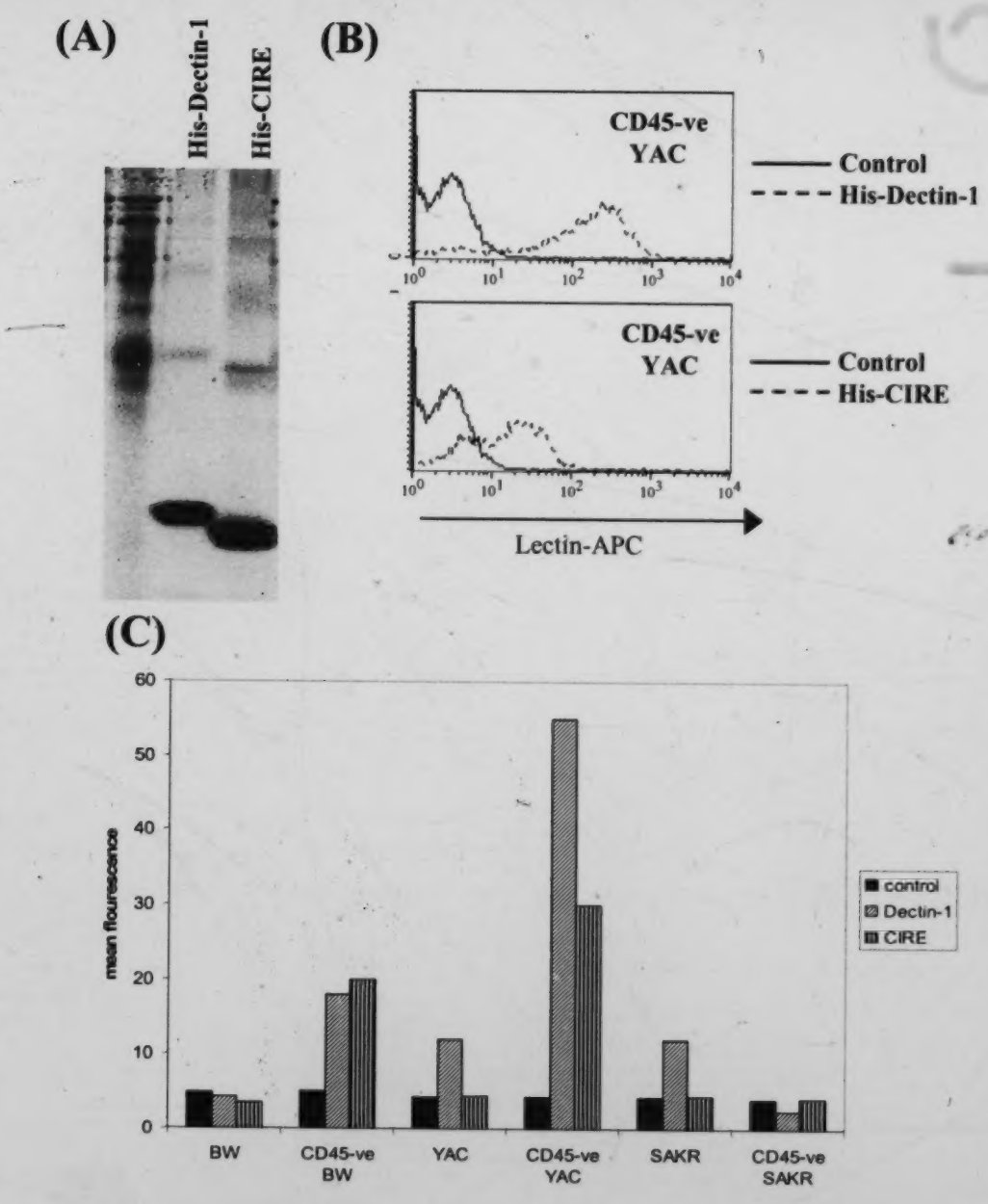


Figure 8: Soluble Dectin-1 and CIRE bind numerous CD45 positive and negative T cell lines. Soluble 6xHis-tagged lectin was purified and run on an SDS-PAGE gel (A) and used in a T cell binding assay as described. Representative binding to YACN1 cells is displayed in (B) and graphed as mean fluorescence in (C) along with binding to other CD45 positive and negative cell lines. This experiment was repeated four times with similar results.

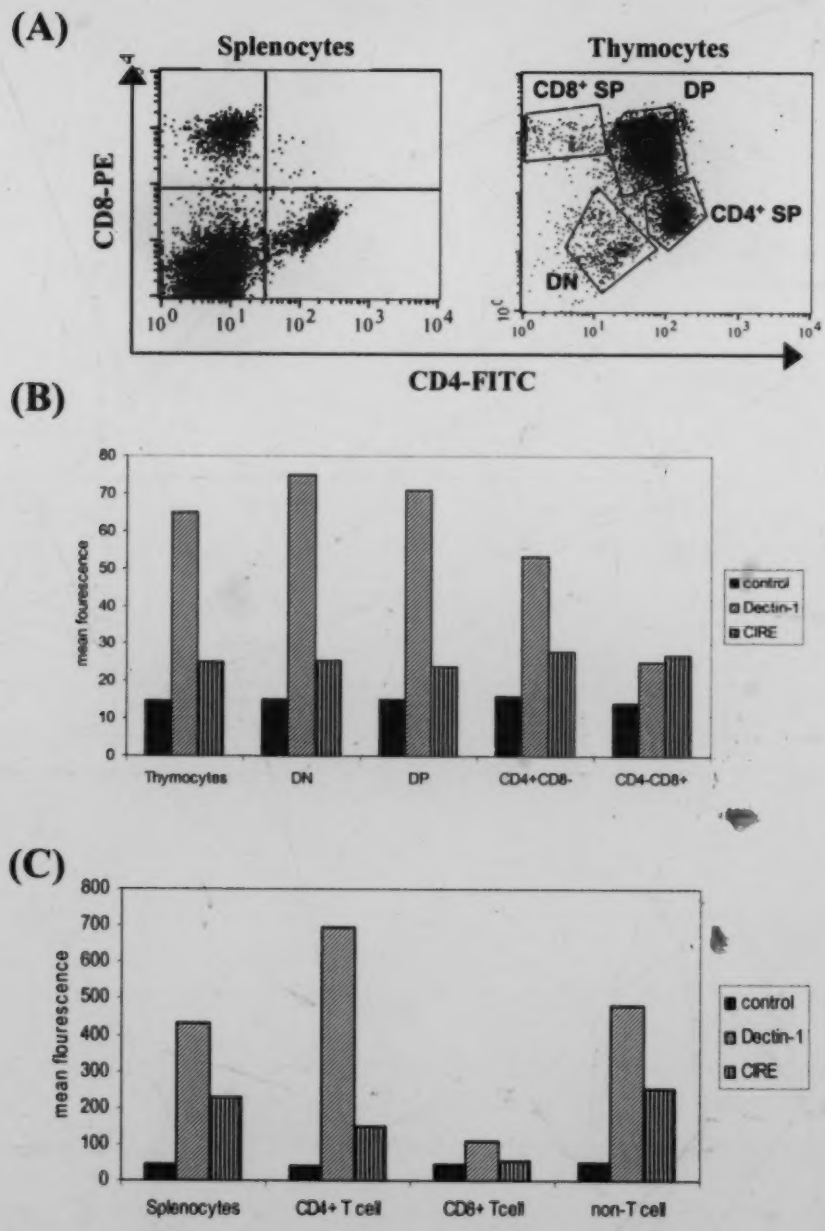


Figure 9: Soluble Dectin-1 and CIRE bind *ex vivo* T cells and Dectin-1 alone binds thymocytes. *Ex vivo* splenocytes and thymocytes were isolated from C57B/6J mice and stained for CD4 and CD8 expression (A). These populations were then evaluated for Dectin-1 and CIRE binding, presented graphically as mean fluorescence in (B) thymocytes and (C) splenocytes. These results were repeated three times with similar results.

displaying significantly weaker binding to $CD8^+$ SP cells (Figure 9B). A similar trend was also observed in splenic T cells where Dectin-1 demonstrated stronger binding to $CD4^+$ T cells than their $CD8^+$ counterparts (Figure 9C). Interestingly, Dectin-1 also appeared to bind a $CD4^-CD8^-$ non-T cell population.

In agreement with the conjugate assay data, CIRE showed little to no binding to thymocytes (Figure 9B), however it did display binding to splenocytes. Furthermore, it bound both $CD4^+$ and $CD8^+$ T cells, with superior binding observed to $CD4^+$ cells, but interestingly, strongest binding was observed to a $CD4^-CD8^-$ non-T cell population (Figure 9C).

To further characterize which T cell populations were being bound, splenic T cells were purified by MACS, and co-staining for the naive T cell marker CD62L, activation marker CD69, memory marker CD44 was performed in addition to CD4, CD8 and lectin staining (Figure 10A). Both lectins demonstrated similar binding preferences and while both bound all populations to some degree, they appeared to display a preference for $CD4^+$ T cells and T cells with an activated phenotype (Figure 10B, 10C and 10D). This data taken together, implicated Dectin-1 in both T cell development and activation, while CIRE likely plays a role only in the latter.

3.6 Dectin-1 and CIRE bind other lymphocyte populations

As mentioned in the previous section, both Dectin-1 and CIRE demonstrated binding to a $CD4^-CD8^-$ splenic lymphocyte population. In terms of lymphocytes, the two populations most likely to comprise these lectin bound cells are NK or B cells. To more closely study the possibility that CIRE and/or Dectin-1 may be binding these cells, I stained freshly isolated splenocytes with lectin and the NK cell marker NK1.1 or the B

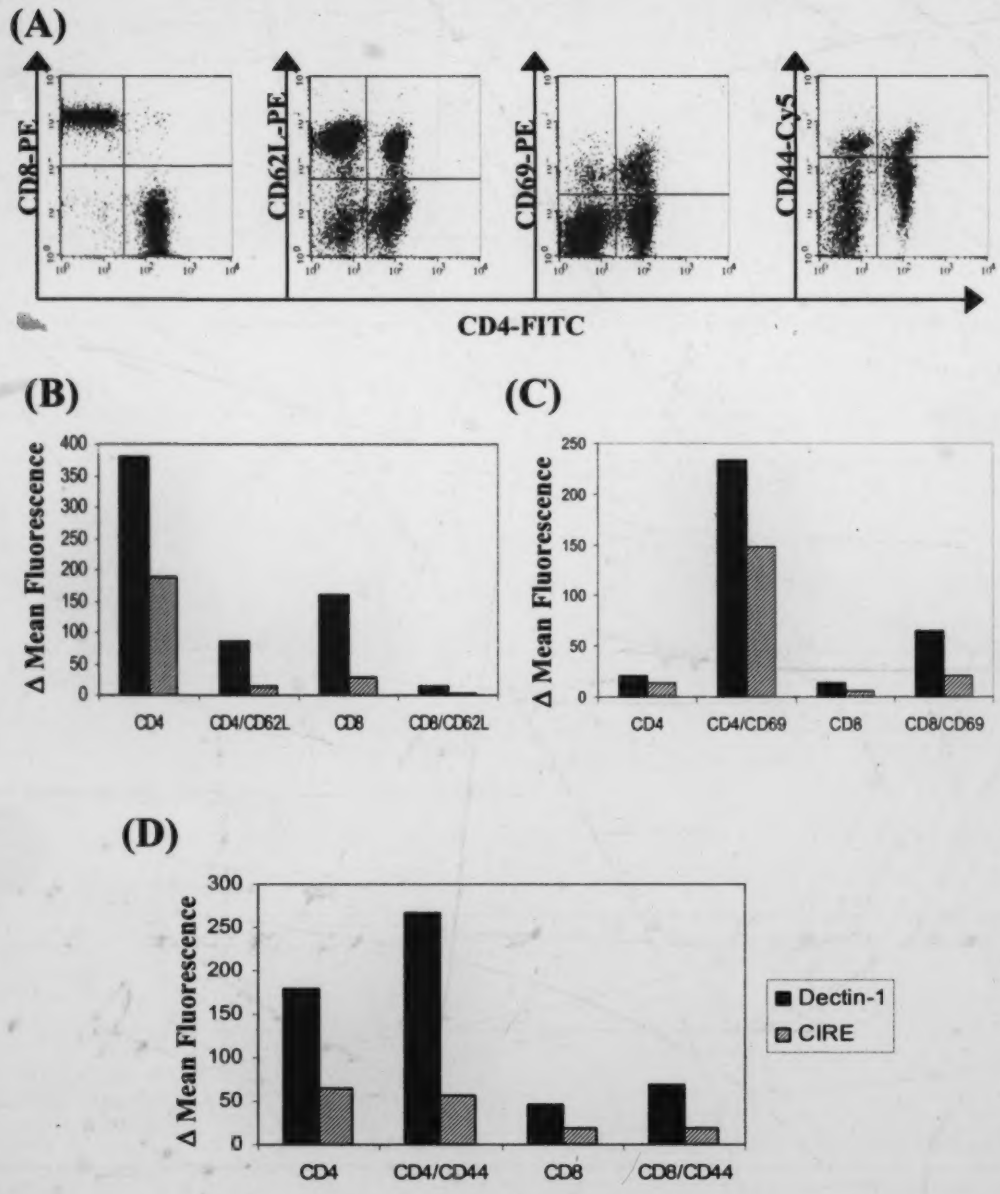


Figure 10: Dectin-1 and CIRE preferentially bind activated T cells. *Ex vivo* T cells were purified from C57B/6J spleen tissue using MACS and stained for CD4, CD8, CD62L, CD69 and CD44 (A). These populations were then evaluated for Dectin-1 and CIRE binding as presented graphically in (B) CD62L, (C) CD69 and (D) CD44. Data is presented as mean fluorescence above negative control, and was repeated two times with similar results.

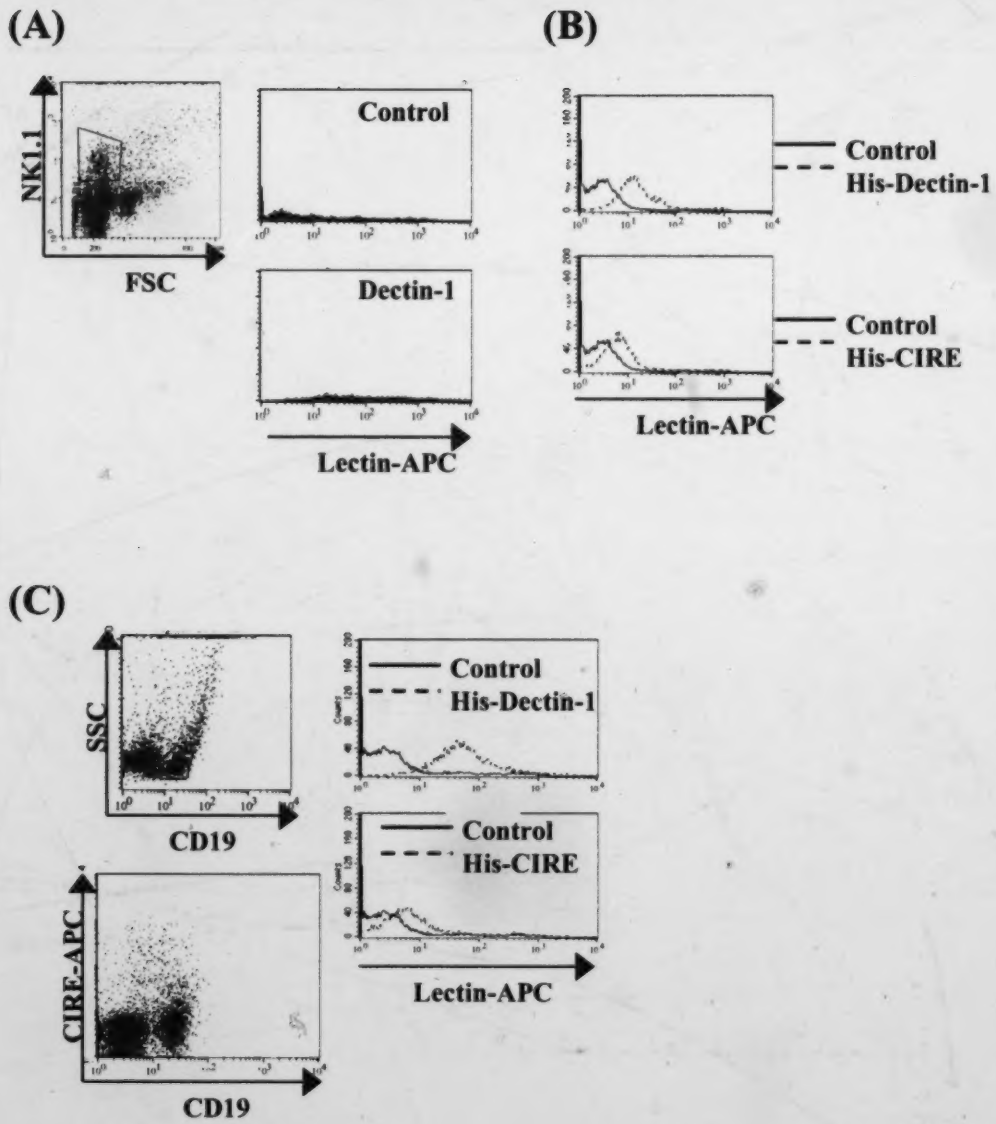


Figure 11: Dectin-1 and CIRE bind other lymphocytes. Lectin binding to NK cells was evaluated by binding to *ex vivo* NK cells though co-staining for NK1.1 (A). Dectin-1 and CIRE were also evaluated for binding to B cells by binding the A20 B cell line (B) and *ex vivo* B cells by co-staining with CD19 (C). This experiment was repeated two times with similar results.

cell marker CD19 (Figure 11A and 11C). While Dectin-1 displayed strong binding to both populations, as well as to the A20 B cell line (Figure 11B); CIRE only bound B cells to any significant extent (Figure 11B and 11C). Furthermore, when CIRE binding is plotted against CD19 staining, CD19 positive B cells bind CIRE to a greater extent than their CD19 negative T cell counterparts, suggesting that B cells account for the CIRE bound $CD4^-CD8^-$ population observed previously (Figure 11C). This data suggests that these lectins may play some as of yet unidentified role in regulation of lymphocytes other than T cells.

3.7 Dectin-1, but not CIRE binding is dependant on calcium

C-type lectins often require coordination of Ca^{2+} ions in order to stably bind carbohydrates, however previous work has shown that Dectin-1 binds yeast independent of metal cations [73, 76]. To determine whether this was also the case for Dectin-1 binding to T cells, as well as to establish the requirements for divalent metal ions for CIRE, lectin staining was performed in the presence of metal chelators. Furthermore, in order to discriminate between any requirement for calcium and other divalent metal cations, staining was done with EDTA (which preferentially chelates Mg^{2+}) plus excess Ca^{2+} or EGTA (preferentially chelates Ca^{2+}) with excess Mg^{2+}. Using this approach I was able to determine that Dectin-1 specifically requires Ca^{2+} for stable binding to the YACN1 T cell line, however CIRE appears to bind YACN1 and BW T cell lines equally in the presence or absence of divalent metal ions (figure 12B and 12C). However, it is also possible that CIRE may coordinate calcium in such a way that chelators such as EDTA/EGTA cannot remove it. This possibility is supported by the observation that CIRE, in addition to Dectin-1, following purification are folded more efficiently in the

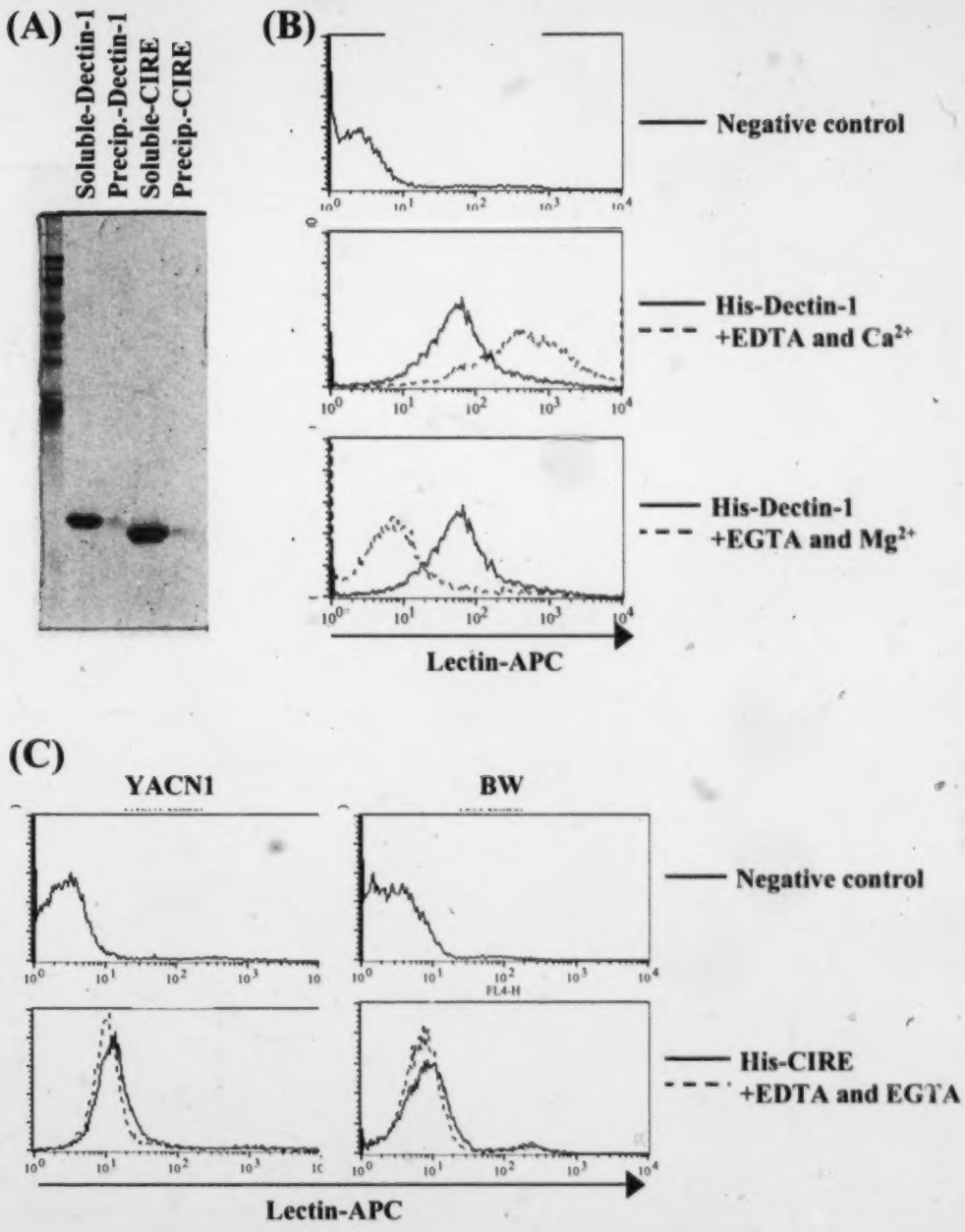


Figure 12: Dectin-1, but not CIRE requires calcium for T cell binding. Dectin-1 binding to YACN1 T cells was performed in the absence of chelators, with 5mM EDTA+5mM $CaCl_2$ or 5mM EGTA+5mM $MgCl_2$ (B). CIRE binding to both BW and YACN1 was done in the presence or absence of 5mMEDTA+5mM EGTA (C). Protein solubility was also verified following addition of chelators as shown in (A).

presence of divalent calcium ions, suggesting that this metal plays a role in the formation of a properly folded CRD (Appendix 1). Additionally, any effects observed following chelation were not due to gross misfolding problems as both lectins remained soluble following removal of calcium and magnesium (figure 12A).

3.8 Dectin-1 recognizes large negative sulfated sugars on T cells

As previously discussed, lectins are often associated with binding to carbohydrates present on proteins or lipids, however the carbohydrate specificity of both CIRE and Dectin-1 binding to T cells remained unidentified. In order to try to answer this question I performed lectin staining either alone or in the presence of sugars in an attempt to block this interaction, thereby providing clues as to the specificity of this interaction. The sugars used were split into panels as follows: 1→Mannose, mannan, mannose-1-phosphate, mannose-6-phosphate, 2→Fructose, fructose-6-phosphate, fucose, sucrose, 3→N-acetyl glucosamine, N-acetyl mannoseamine, N-acetyl galactosamine, 4→Galactose, galactose-6-phosphate, alpha-lactose, beta-lactose, 5→Glucose, Glucose-6-phosphate, Dextrose, Dextran, 6→Barly glucans, laminarin, pullulan, Cellulose, 7→Fucoidin, Galactose-6-sulfate, Glucose-6-sulfate. Unfortunately due to the relatively weak binding observed for CIRE, it was not possible to determine which sugars did and did not have significant effects on binding. For Dectin-1 I found that β-1,3-linked glucans including laminarin (panel 6) which potently inhibits binding to yeast, had no effect on T cell binding as had been previously reported [75] (Figure 13A). However, panel 7 containing the sugars fucoidin, glucose-6-sulfate and galactose-6-sulfate reduced binding of T cells to background levels (figure 13A). When added separately I found that fucoidin was primarily responsible for the observed effect, in that it blocked Dectin-1

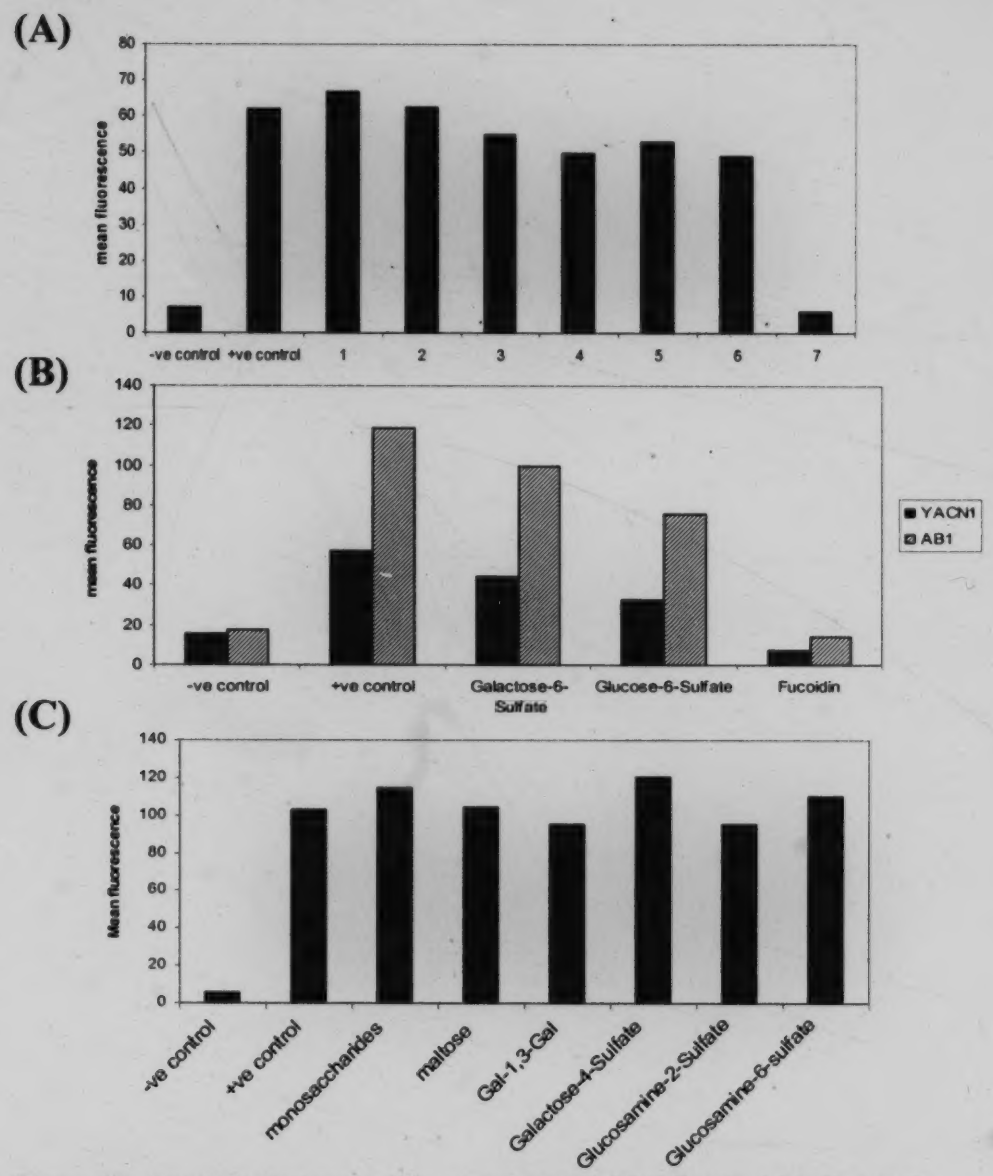


Figure 13: Fucoidin, but none of its components inhibit Dectin-1 binding to T cells. Sugar inhibition of Dectin-1 binding was done by pre-incubation of Dectin-1 with one or more carbohydrates at 100μg/ml on ice. Dectin-1 binding was then evaluated to YACN1 cells as displayed graphically in (A). Panel 7, containing Galactose-6-Sulfate, Glucoase-6-Sulfate and Fucoidin was found to block Dectin-1 binding and so these were evaluated separately for their ability to block binding to YACN1 and AB1 T cells (B). As fucoidin was primarily responsible for this effect, other sulfated sugars or oligosaccharides containing similar linkages were also tested (C). Experiments were repeated twice with similar results.

binding to both YACN1 and AB.1 T cells (Figure 13B). Fucoidin is a large, heavily sulfated sugar composed mostly of α-1,3-linked- and α-1,4-linked fucose, but also contains glucose, mannose, galactose, xylose and rhamnose. When these monosaccharides or other disaccharides with α-1,3- or α-1,4- linkages (maltose, Galactose-1,3-Galactose) where evaluated, none where found to have a significant effect (Figure 13C). Similarly no other simple sulfated sugars (Galactose-4-Sulfate, glucosamine-2-sulfate, glucosamine-6-sulfate) resulted in any significant inhibition of Dectin-1 binding (Figure 13C). However, a variety of other large sulfated sugars including chondroitin sulfate A, chondroitin sulfate B, heparin, heparin sulfate and dextran sulfate all potently blocked binding, suggesting that Dectin-1 has a preference for large, sulfated, negatively charged sugars (Figure 14A). However other closely related sugars including chondroitin sulfate C, glucosamine-2,6-sulfate and hyaluronate had no effect, suggesting that there are also some specific requirements for stable Dectin-1 binding (Figure 14A). Interestingly, although β-glucans such as laminarin have no effect on T cell binding, fucoidin completely blocked both T cell and yeast binding to Dectin-1, arguing against the suggestion that Dectin-1 has two separate binding sites (Figure 14B) [179, 187].

To more directly establish whether Dectin-1 bound a sulfated ligand on T cells I studied binding of Dectin-1 in the presence or absence of sodium chlorate, which competitively blocks formation of 3'-phosphoadenosine-5'-phosphosulfate (PAPS), the high energy donor in cellular sulfation reactions. I performed this on the NZB T cell line as it had been previously used in similar studies looking at sulfation of MHC class I, and whose cell surface sulfation is known to be potently blocked by sodium chlorate [209].

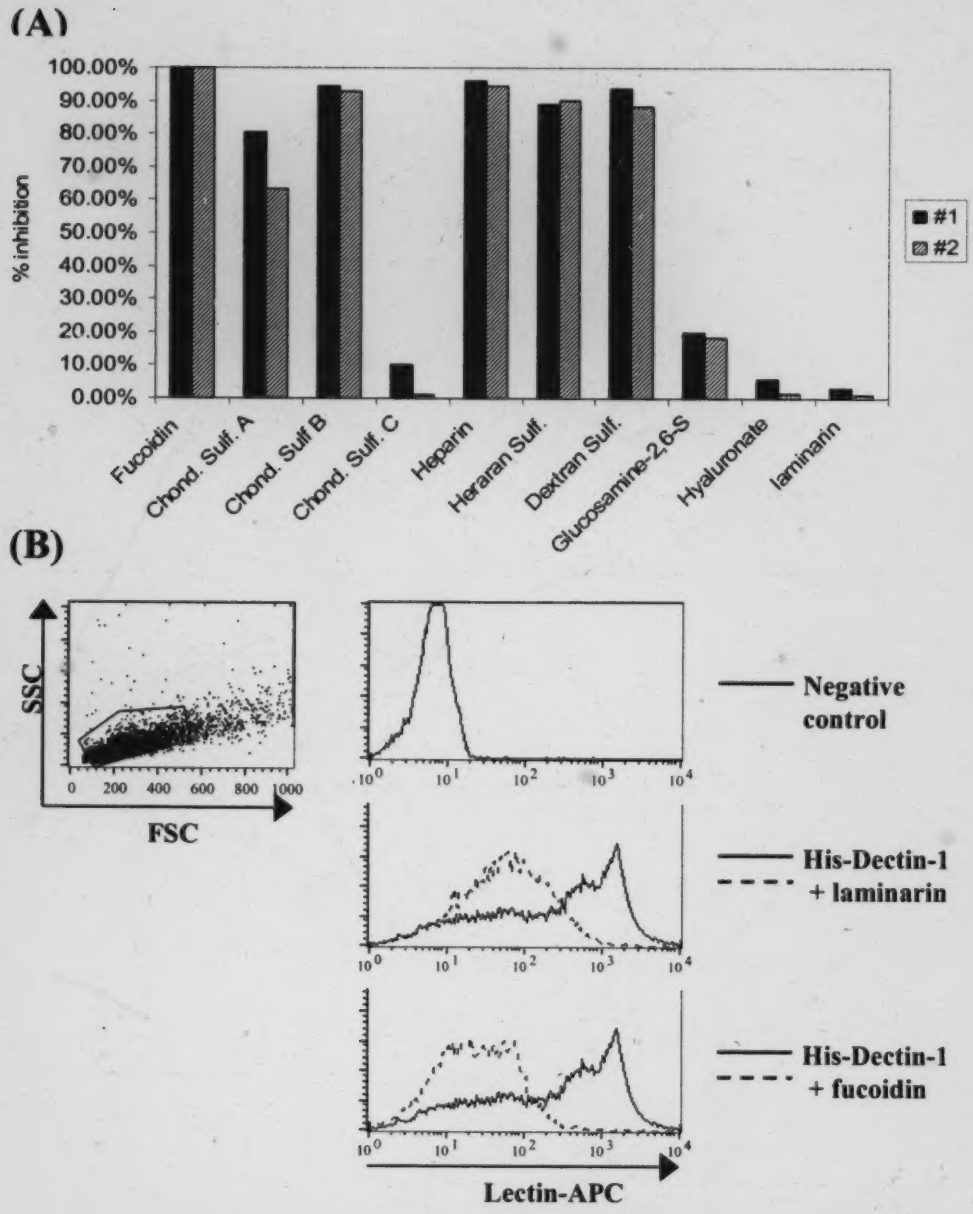


Figure 14: Multiple large sulfated sugars inhibit Dectin-1 binding to T cells and yeast. In addition to fucoidin, other related large negatively charged sulfated sugars were also added at 100μg/ml in an attempt to block Dectin-1 binding to T cells (A), two representative experiments are shown. Data is presented as % inhibition= [(positive control-negative control)/(sample mean fluorescence-negative control)]x100%. Ability of Fucoidin to block binding to the known Dectin-1 target, zymosan, as compared to laminarin was also tested (B). Experiments were repeated three times with similar results.

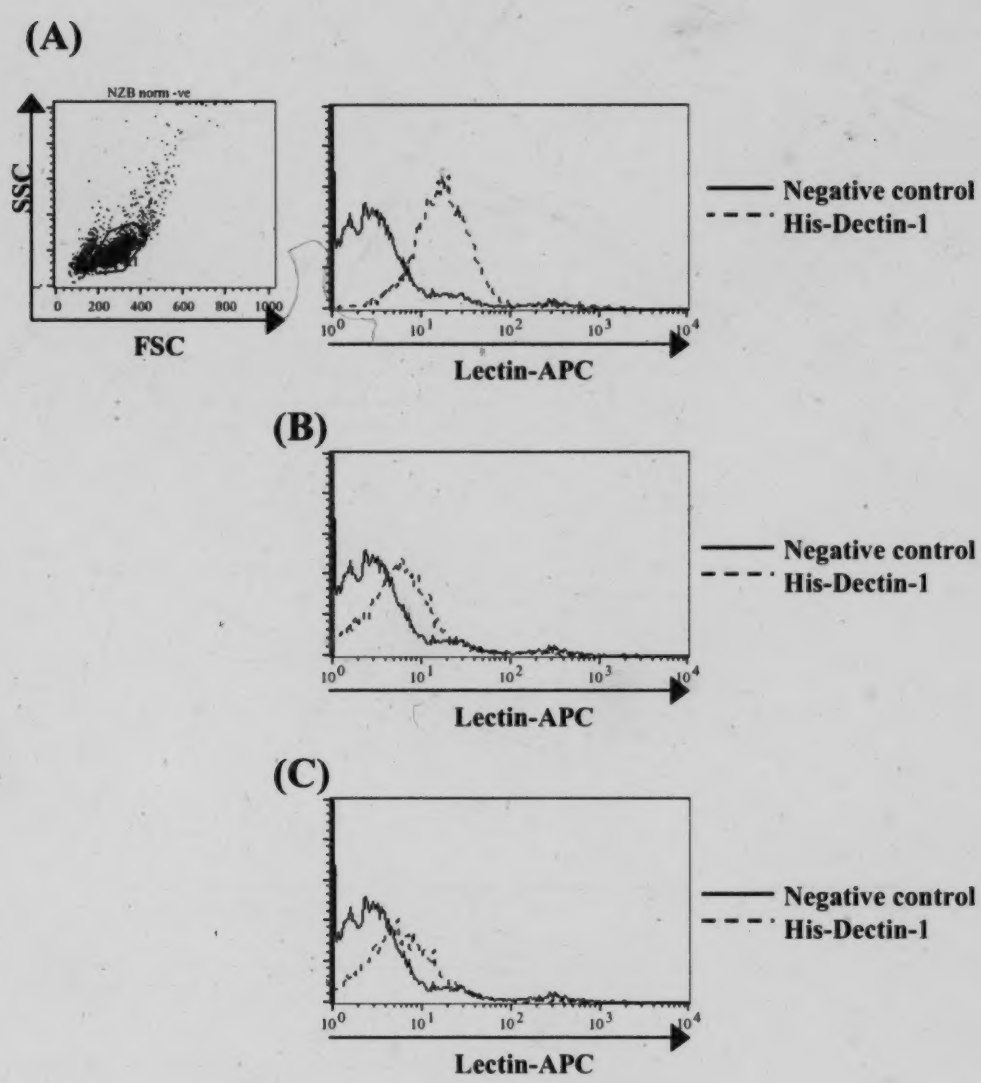


Figure 15: Dectin-1 binds a sulfated ligand on T cells. Cell surface sulfation was blocked on YACN1 cells by incubation in two different sulfate free media, Fisher media (B) and Sulfate-free DMEM (C), both of which were supplemented with sodium chlorate for 48 hours. Dectin-1 binding was then tested and compared to control samples with normal sulfation (A). Experiment was repeated twice with similar results.

I found that upon incubation in sulfate-free media supplemented with sodium chlorate, binding to Dectin-1 was significantly reduced as compared to the control, thus suggesting that Dectin-1 does bind a sulfated ligand on T cells (Figure 15).

3.9 Coordination of calcium alters Dectin-1 specificity

The data presented above suggest that Dectin-1 does in fact bind yeast and T cells through a similar site, as large sulfated sugars such as fucoidin can potently inhibit both interactions. However, my data and that of others suggest that binding to yeast is calcium independent whereas binding to T cells absolutely requires this divalent metal cation [76]. When taken together, this suggests that Dectin-1 may bind β-glucans found on yeast in a calcium independent fashion, whereas coordination of calcium within this same site confers the ability to specifically recognize sulfated sugars on T cells. To more closely examine this, I studied binding of Dectin-1 to zymosan, a yeast derived particle, previously seen to bind Dectin-1 with high affinity [179]. I studied the ability of both laminarin and fucoidin to block binding to zymosan in the presence or absence of calcium through the addition of EGTA. As before, EGTA has no effect alone, whereas both laminarin and fucoidin both blocked Dectin-1 binding to a similar degree (Figure 16B and 16C). Upon removal of calcium with EGTA, laminarin retained its ability to block binding suggesting that this β-glucan can occupy the Dectin-1 CTLD in the absence of calcium. In contrast, fucoidin lost its ability to inhibit Dectin-1 binding to zymosan upon addition of EGTA, suggesting that binding of sulfated sugars requires calcium and that by removing this ion the specificity of Dectin-1 is altered as to only allow binding of β-glucans (Figure 16B and 16C).

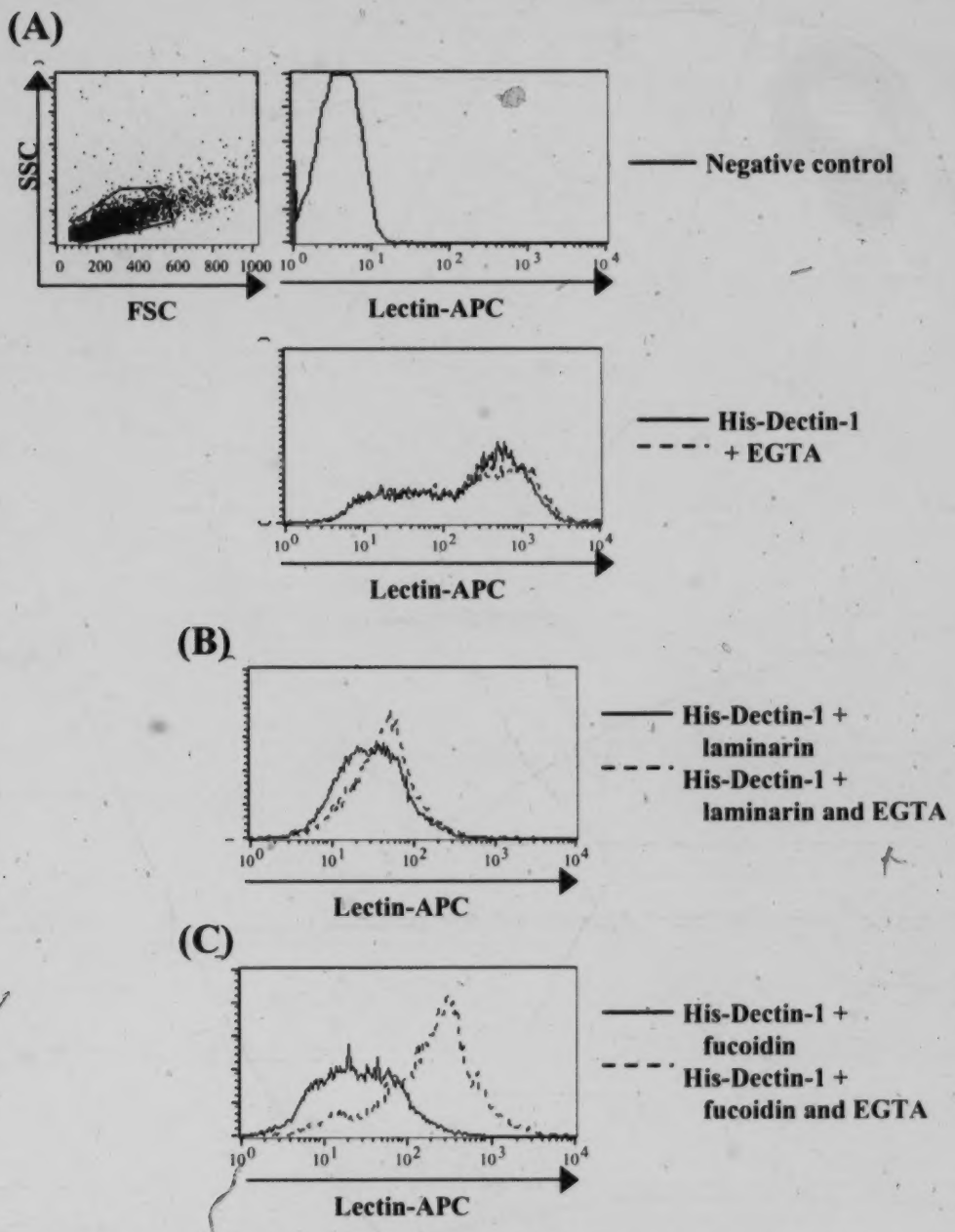


Figure 16: Presence of calcium alters Dectin-1 binding specificity. Binding of Dectin-1 to β-glucans on the surface of the yeast derived particle, zymosan, was evaluated in the presence or absence of 5mM EGTA (A), 100mg/ml Laminarin (B), with 100mg/ml Fucoidin (C), Laminarin+EGTA (B) or Fucoidin+EGTA (C). Shown above is a representative experiment, which was repeated three times with similar results.

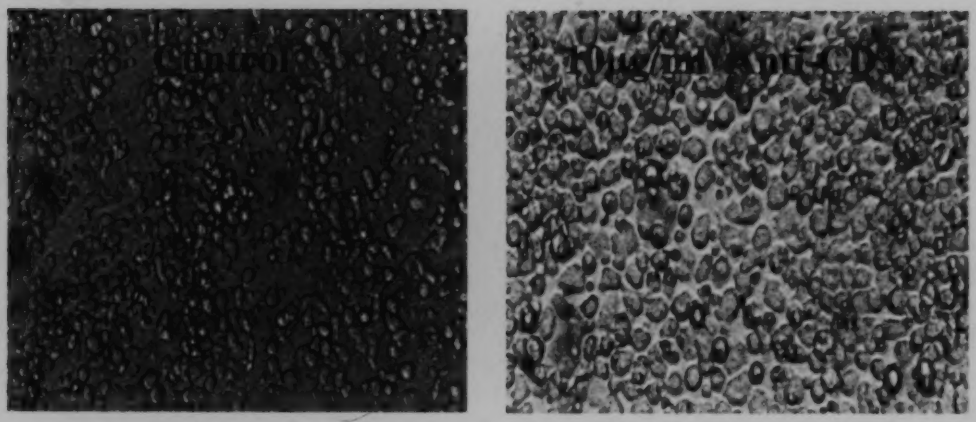
3.10 CIRE and Dectin-1 enhance CTL spreading on anti-CD3

Once the physical binding properties of these lectins had been characterized, I next wanted to determine what, if any, effect did this binding have with regards to T cell activity or signaling. Unlike CIRE for which nothing is known, Dectin-1 has already been proposed to have some degree of co-stimulatory activity upon binding to T cells [76, 174]. To initially assess what effect these lectins had following T cell binding, I first looked at their ability to enhance or inhibit spreading of the AB.1 CTL clone in response to full or sub-optimal stimulation through CD3. Co-immobilization of lectins with anti-CD3 revealed that both Dectin-1 and CIRE could enhance spreading when combined with sub-optimal stimulation through CD3, but neither had any inhibitory effect nor could elicit any effect alone (Figure 17A and 17B). This finding supports a position for Dectin-1 as a T cell co-stimulatory molecule and suggests that CIRE may play a similar role.

3.11 CIRE and to a lesser extent Dectin-1 enhance CTL degranulation in response to anti-CD3

Another hallmark of CTL activation is degranulation which can be readily monitored by analyzing serine esterase release [208]. Since I had already seen an effect of both CIRE and Dectin-1 on spreading, I next sought to evaluate whether lectins could trigger degranulation of CTL on their own or when combined with suboptimal quantities of anti-CD3. Similar to spreading, CIRE dramatically enhanced degranulation, even with anti-CD3 concentrations down to 0.01μg/ml. Dectin-1 was also observed to enhance degranulation, however only at somewhat higher anti-CD3 concentrations (0.1μg/ml) and not to the same extent as CIRE, which fully restored degranulation to levels observed

(A)



(B)

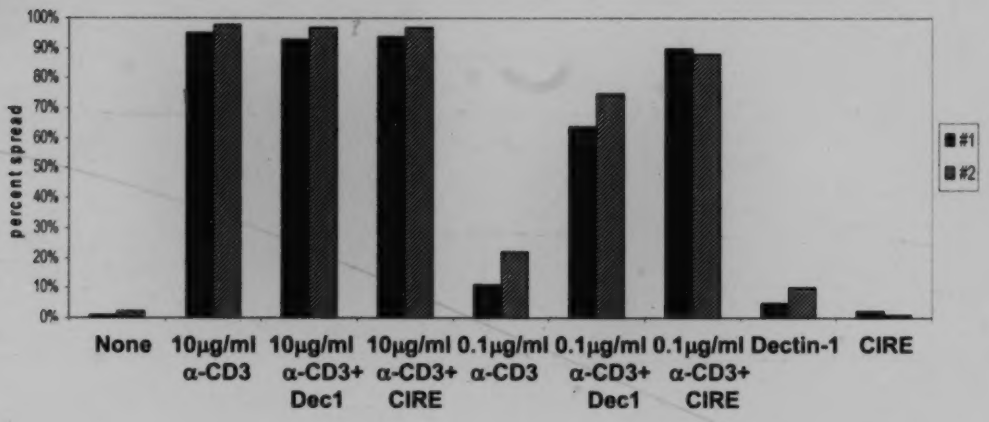


Figure 17: Dectin-1 and CIRE enhance T cell spreading in response to anti-CD3. AB1 clones were stimulated with varying concentrations of anti-CD3(2C11) with or without 15μg/ml Dectin-1 or CIRE, or with lectins alone. Following 30 minutes cell were visualized, representative controls are shown in (A) and data is presented graphically in (B) as % of cell spread. Experiment was repeated five times with similar results.

under full activation (figure 18). However, neither lectin had any effect alone. This data indicates that Dectin-1 and especially CIRE may act to augment signals sent through the TCR, supporting a potential co-stimulatory role for these two lectins.

3.12 CIRE and Dectin-1 enhance IFN-γ production from CTL in response To anti-CD3

Upon activation of CTL, including the AB.1 clones, these cells often are observed to release a variety of cytokines, of which IFN-γ is one of the most important. Both CIRE and Dectin-1 had been observed to augment other aspects of T cell activation, so I next decided to evaluate whether they could also enhance IFN-γ production using a standard sandwich ELISA approach. Briefly cells were stimulated for 24 hours on immobilized lectins, with or without suboptimal quantities of anti-CD3, at which point media was collected and analyzed for IFN-γ using a sandwich ELISA. I found that both lectins could similarly enhance IFN-γ secretion together with suboptimal amounts of anti-CD3, once again suggesting a potential co-stimulatory role (Figure 19). This also confirms previous work on Dectin-1 that suggests it could enhance IFN-γ release [174].

3.13 CIRE, but not Dectin-1 increase tyrosine phosphorylation within CTL in response to anti-CD3

The above studies indicate that both Dectin-1 and CIRE could elicit numerous effects indicative of T cell activation when co-immobilized with sub-optimal amounts of anti-CD3, this suggested that they may be in some way triggering signaling within these T cells. Upon initiation of signaling in T cells, multiple kinases become activated leading to the phosphorylation of numerous intracellular signaling proteins. Much of this

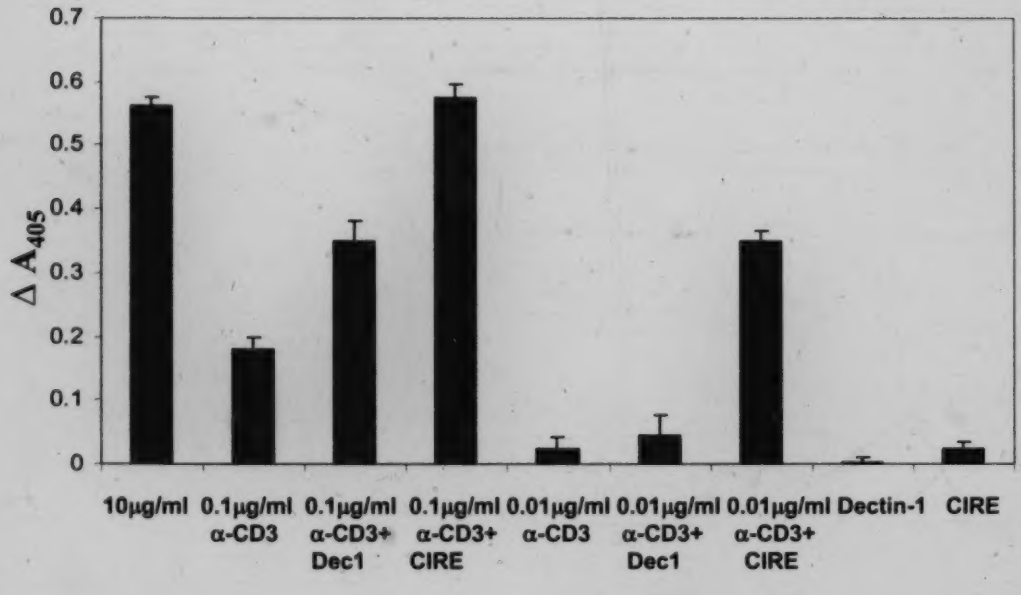


Figure 18: Dectin-1 and CIRE enhance T cell degranulation in response to anti-CD3. AB1 clones were stimulated with varying concentrations of anti-CD3(2C11) with or without 15μg/ml Dectin-1 or CIRE (15μg/ml), or with lectins alone. Following 4 hours media was collected and used in a serine esterase assay. Data is presented graphically as change in absorbance at 405nm as compared to negative control. Data represents average of five experiments.

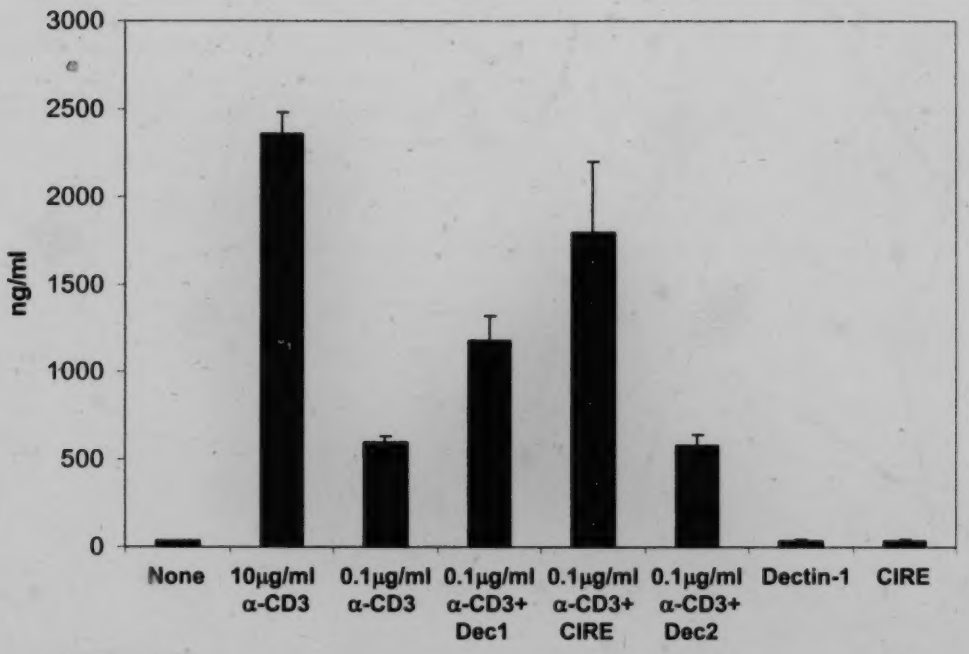


Figure 19: Dectin-1 and CIRE enhance IFN-γ release from T cells in response to anti-CD3. AB1 clones were stimulated with varying concentrations of anti-CD3(2C11) with or without 15μg/ml Dectin-1 or CIRE, or with lectins alone. Following 24 hours supernatants were collected and used in an IFN-γ ELISA and compared against a standard. Data is presented graphically and represents an average of four experiments.

phosphorylation occurs on tyrosine residues, and these tyrosine phosphorylation events can be readily detected by western blot analysis using anti-phosphotyrosine antibodies. To study how potent the observed co-stimulatory effects of these lectins are, I stimulated AB.1 clones for 20 minutes with lectin while titrating down the quantity of anti-CD3. While Dectin-1 had little noticeable effects in terms of increasing tyrosine phosphorylation as compared to the control; which had effects down to 0.04μg/ml (figure 20A and B). CIRE had potent effects down to 0.001μg/ml, once again confirming that it may act in a co-stimulatory fashion (Figure 20C, 20D and 20E).

3.14 Search for CIRE and Dectin-1 ligands

To this point I had observed binding of both CIRE and Dectin-1 to multiple T cell lines and *ex vivo* T cells and seen that this binding can potentially modulate T cell responses in a co-stimulatory type fashion. So the next question I wanted to answer was what ligand(s) are these lectins binding on the surface of T cells and given my original hypothesis, does CD45 play any role in this interaction? In order to answer this I utilized the Sulfo-SBED chemical cross-linker (Sigma Chemicals, St. Louis, MO), which was initially conjugated to the soluble CIRE or Dectin-1 constructs and then following incubation on YAC T cells was UV cross-linked to any potential ligands. This linkage was then severed under reducing conditions resulting in the attachment of a biotin residue onto the target proteins; these proteins were then subsequently separated by SDS-PAGE, transferred and detected by streptavidin-HRP. Although no specific ligands could be detected for Dectin-1, one specific band of approximately 80-85kDa was detected for CIRE suggesting the existence of a specific ligand for this lectin on the surface of this T cell line (Figure 21). Furthermore, blotting with anti-CD45 antibodies revealed no

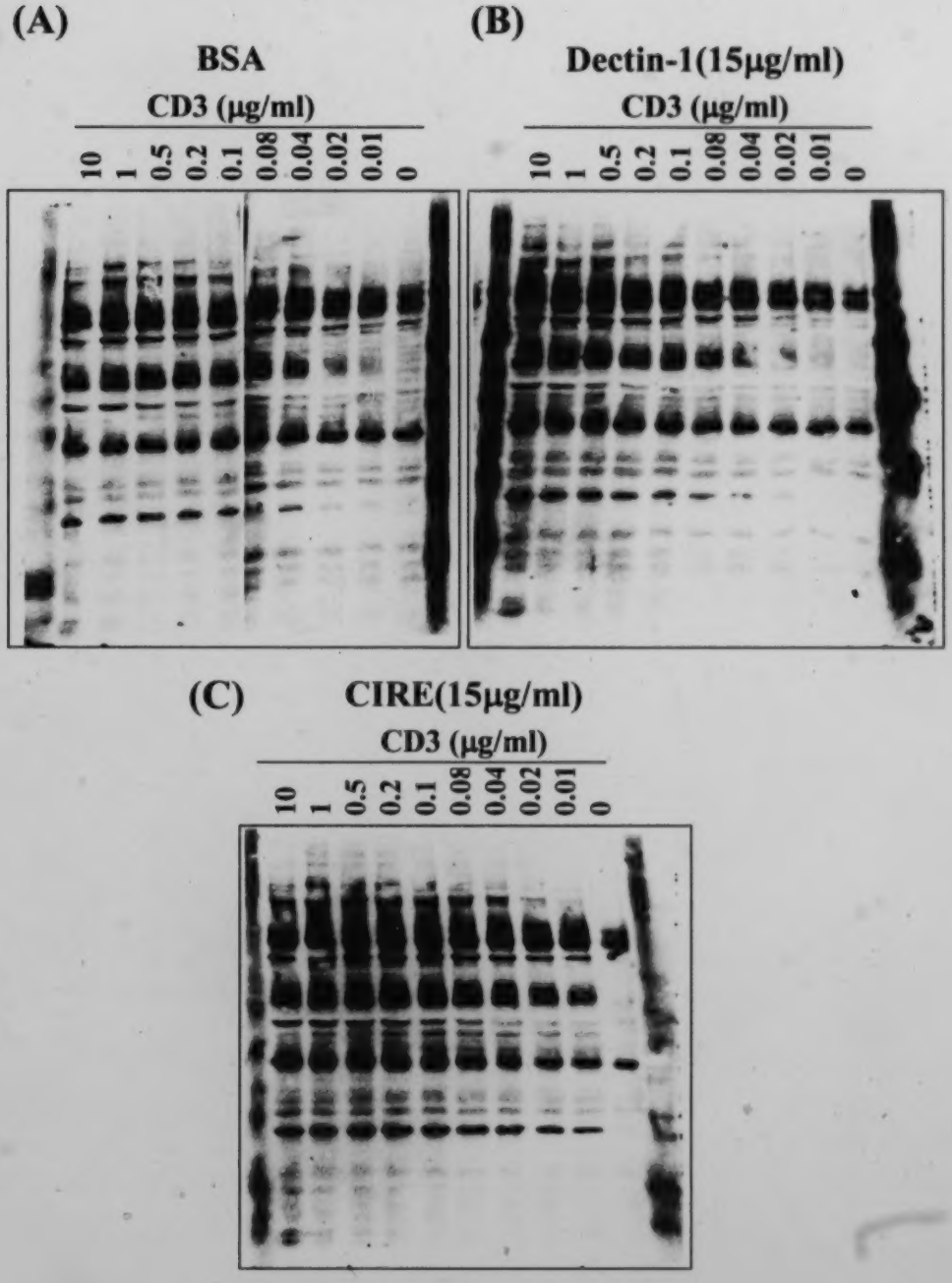


Figure 20: CIRE enhances tyrosine phosphorylation of T cell proteins in response to anti-CD3. AB1 clones were stimulated for 25 minutes with varying concentrations of anti-CD3(2C11) alone (A) or with 15μg/ml Dectin-1(B) or CIRE (C). Reduced whole lysates were blotted with phospho-tyrosine antibody.

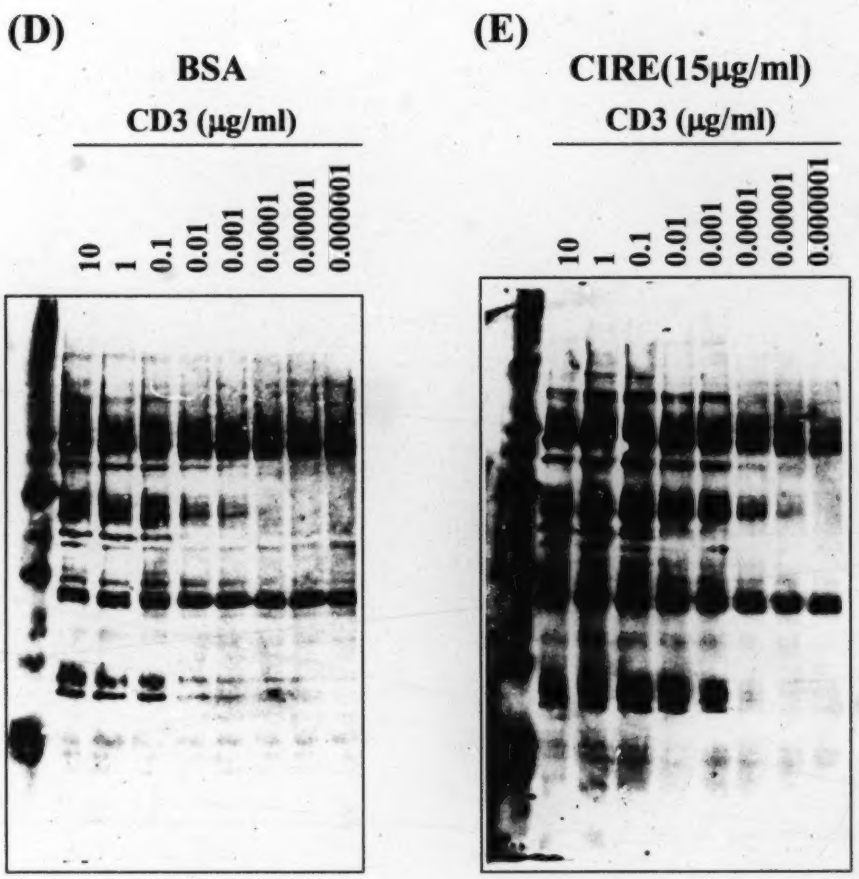


Figure 20: AB1 clones were stimulated for 25 minutes with lower concentrations of anti-CD3(2C11) alone (D) or together with CIRE (E). Reduced whole lysates were blotted with phospho-tyrosine antibody.

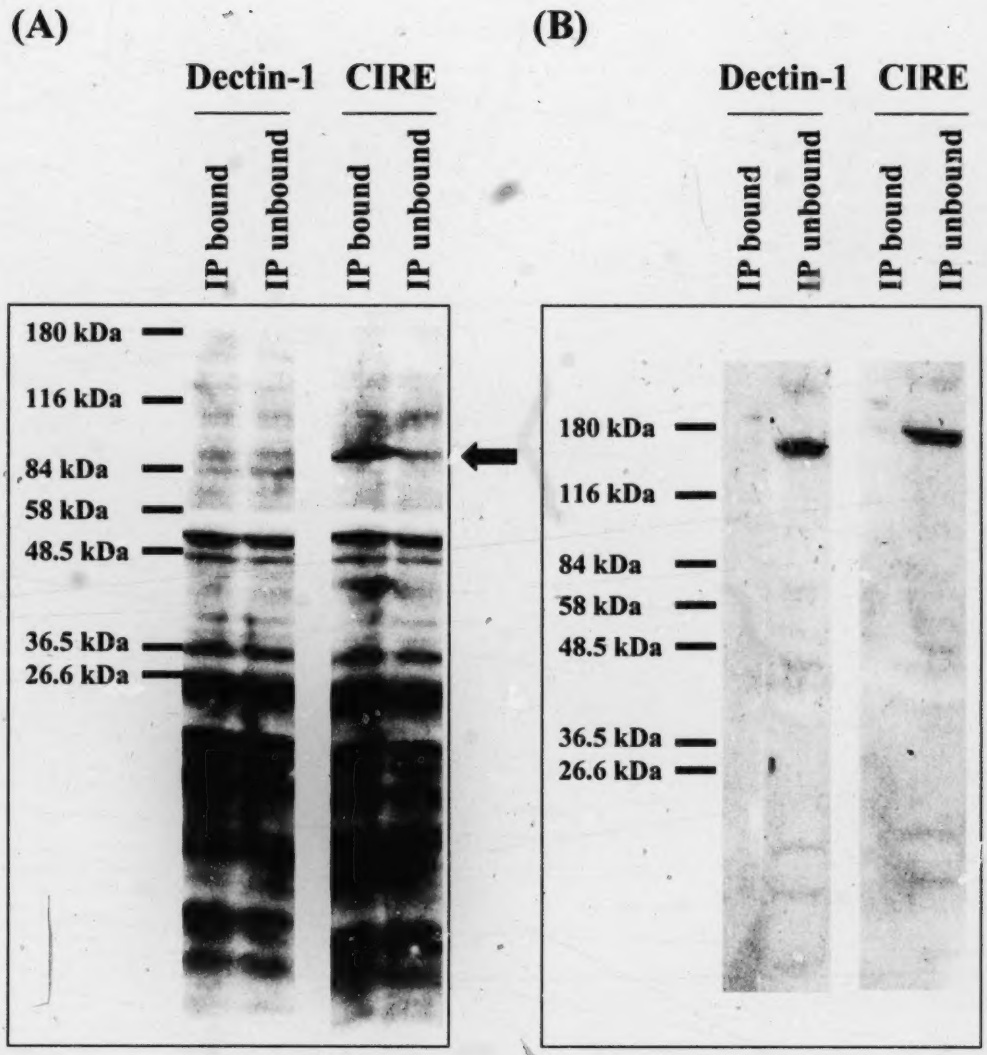


Figure 21: CIRE binds a protein of 85KDA on the T cells surface. (A) Cells were incubated with Sulfo-SBED coupled lectin for 40 minutes on ice followed by UV induced crosslinking. Biotinylated complexes were then isolated using streptavidin coupled beads and separated by SDS-PAGE under reducing conditions. Biotin labeled proteins were then detected by western blot analysis using streptavidin. (B) Blot was stripped and re-probed with anti-CD45 to determine whether either lectin was capable of recognizing this PTPase.

significant binding to CD45 under these conditions (Figure 21B), although this does not rule out the possibility that CD45 may be involved under different conditions or on different T cell populations.

3.15 Summary

The results presented within this chapter lead me to conclude that both CIRE and Dectin-1 are capable of binding T cells and through this interaction they are able to enhance signals provided through the TCR, however they have no effects when bound alone. Dectin-1 is present in both the thymus and spleen and binds immature thymocytes in addition to mature T cells suggesting it may play a role in both development and activation, whereas CIRE only binds mature T cells suggesting its primary role may be in activation of mature T cells alone. Both these lectins were also seen to bind other lymphocyte populations, however the functional significance of this remains unknown. I also briefly studied Dectin-2, and although present in the thymus and lymph node, I was unable to detect any binding to any of the developing thymocyte or T cell populations.

CHAPTER IV

ANTI-CD45 INDUCED EFFECTS

4.1 Introduction

Since I intended to characterize the effects of ligand binding to CD45, and a specific CD45 ligand has not been identified, I adopted an approach that utilized anti-CD45 monoclonal antibodies to mimic ligand binding. The response of T cells to immobilized anti-CD45 antibody have been previously characterized [165, 166]. However with regards to binding of soluble anti-CD45 antibodies, although these have been observed to elicit effects including clustering, the mechanism of this remains poorly understood [167-169]. Furthermore, many discrepancies existed between studies looking at binding of different anti-CD45 antibodies, suggesting that binding of different epitopes within the CD45 extracellular domain may have differential effects with regards to T cell function. In order to further understand these epitope specific effects, I compared multiple monoclonal CD45 antibodies with regards to their effects on T cells and attempted to further characterize how they differed in terms of the mechanism used to elicit these effects.

4.2 Anti-CD45RB mAb induce homotypic T cell clustering

Soluble antibodies to CD45 have been observed to elicit a number of effects on T cells, the most studied of which is cell-cell adhesion in both a homotypic and heterotypic fashion [167-169]. To determine whether our CD45 antibodies would induce a similar response, soluble I3/2 (pan-CD45) or MB23G2 (anti-CD45RB) were added to BW cells without secondary cross-linking, for 30 minutes. Cells were then visualized by light microscopy to study whether or not homotypic clustering was occurring. Addition of

either antibody significantly increased clustering of BWs, suggesting both were capable of inducing cell-cell adhesion, through an unidentified mechanism (figure 22A-C). Interestingly, although both antibodies are equally capable of binding this cell line, addition of the anti-CD45RB antibody MB23G2 resulted in more rapid and intense clustering as compared to the pan-specific I3/2 antibody.

4.3 Anti-CD45 antibodies differ in ability to induce clustering versus spreading

Previous work in our lab suggested that antibodies specific for CD45, including I3/2, when bound to T cells could trigger activation of numerous intracellular signaling molecules resulting in rearrangements of the cytoskeleton as seen by spreading of the cells on plastic [165, 166]. Due to the wide range of results obtained from many of the studies using specific anti-CD45 antibodies, it has been suggested that binding or cross-linking of different epitopes on the CD45 extracellular domain may elicit different responses. To directly study this, I analyzed a panel of different CD45 monoclonal antibodies, including I3/2 (pan-specific). M1/9, M1/89, MB23G2 (anti-CD45RB) and MB4B4 (anti-CD45RB), and studied their ability to induce either adhesion when immobilized to plastic (Figure 23A) or clustering when added to solution (figure 23B). Whereas I3/2 appeared more capable of inducing cell spreading, other antibodies including M1/9, MB23G2 and MB4B4 were superior at inducing clustering. Although M1/89 bound to T cells (data not shown), it induced neither cell spreading, nor clustering (Figure 23). It should also be noted that MB23G2 is not capable of binding the YAC T lymphoma and so was unable to elicit any effect on this cell line. Similar results were obtained from all three T cell lines/clones studied including the BW and YAC lines and AB.1 clones. These quantitative differences in the ability of these antibodies to elicit

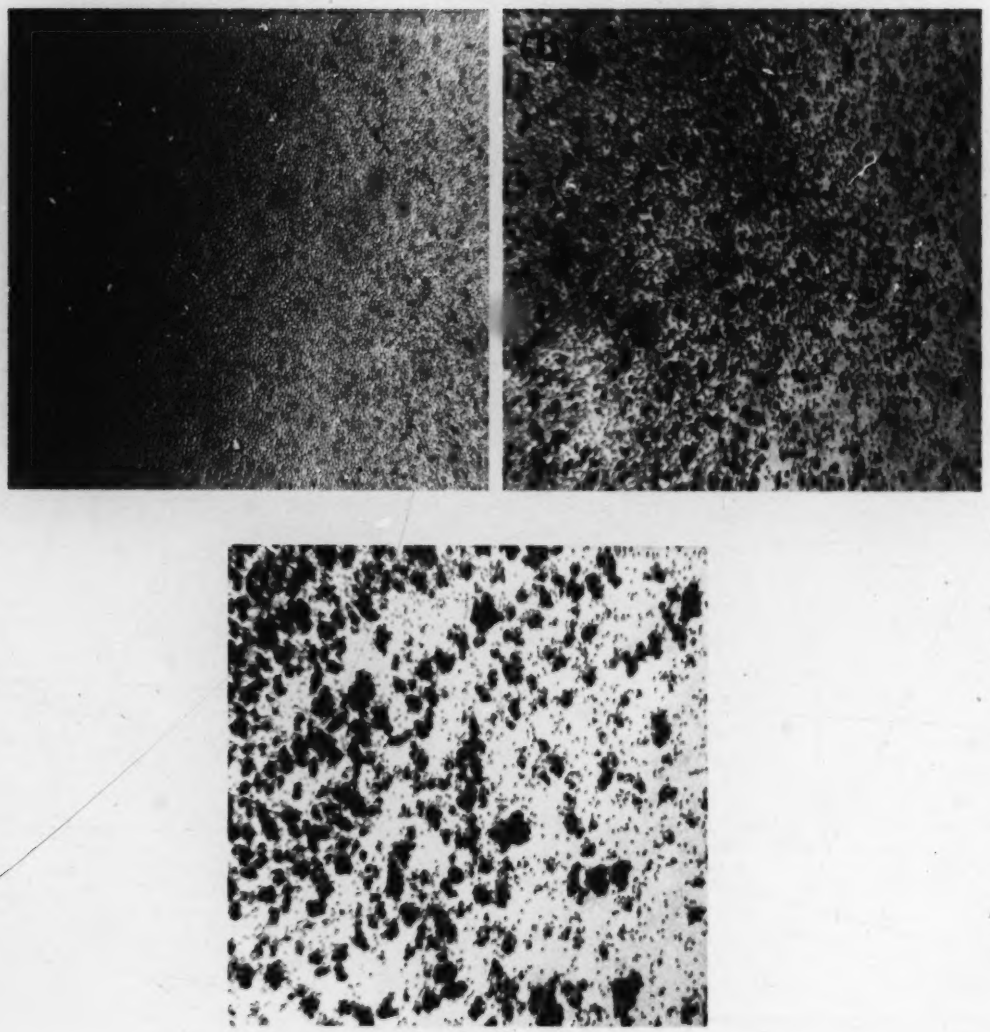


Figure 22: Anti-CD45 antibodies induce homotypic T cell clustering. BW T cells were incubated alone (A) or with either soluble I3/2 (B) or MB23G2 (C) anti-CD45 antibodies for 30 minutes and subsequently visualized by light microscopy. Experiment was repeated four times with similar results.

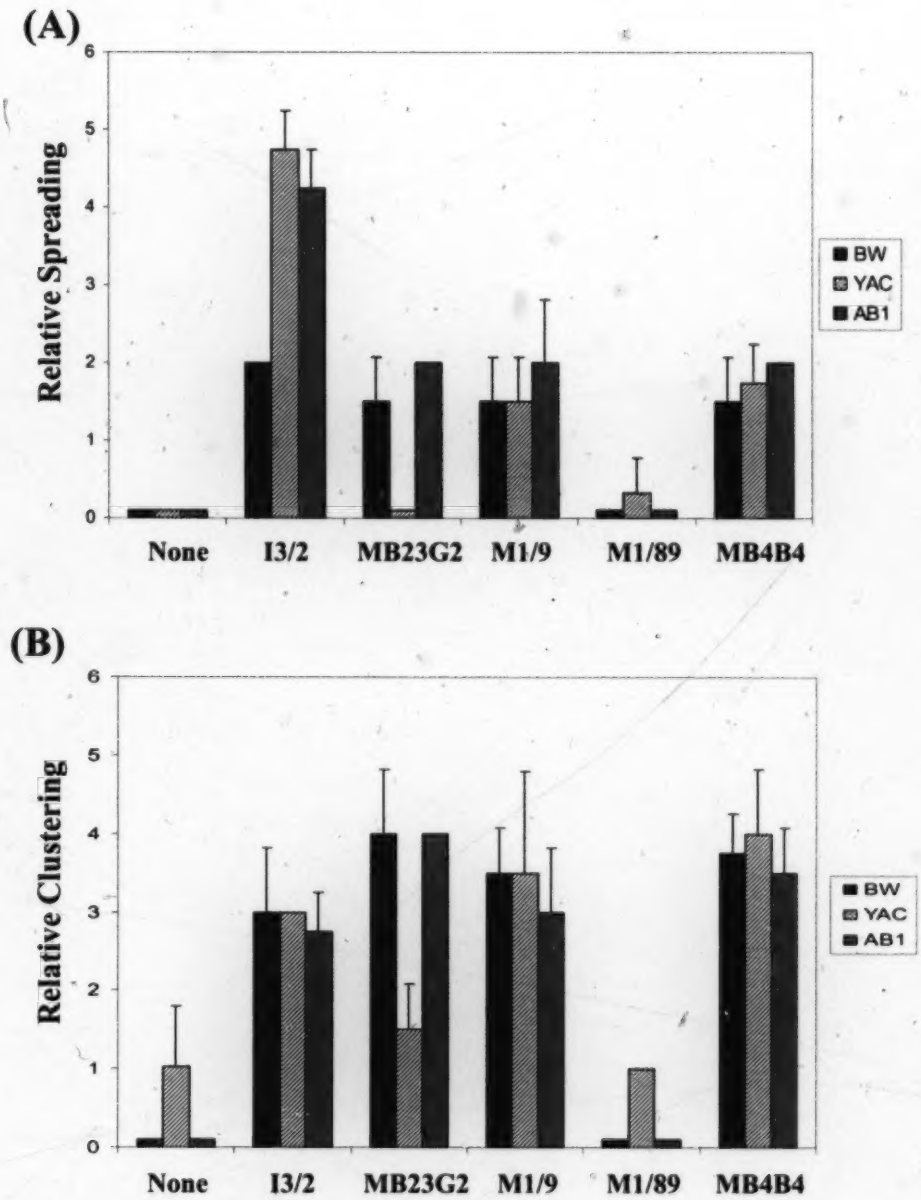


Figure 23: Different CD45 antibodies elicit different responses in T cells. BW, YAC or AB1 T cells were incubated with immobilized (A) or soluble (B) anti-CD45 antibodies and monitored for adhesion and clustering respectively, as described. Data is presented in graphical form with 0 corresponding to no response and 5 to a strong response. This data represents an average of four experiments.

these different effects when bound to the same molecule on the T cell surface confirms that epitope specific effects may in fact exist with regards to binding of the CD45 extracellular domain and the resulting signaling.

4.4 Pan-CD45 antibody Fabs but not anti-CD45RB antibody Fabs induce clustering

Of the antibodies studied above, binding of I3/2 elicited the best spreading, while MB23G2 induced the strongest clustering and so these two antibodies were selected for use in the remainder of this study. One important question that remained unanswered from previous studies looking at T cell clustering resulting from binding of anti-CD45 was whether this effect required cross-linking of the extracellular domain, or if perhaps binding of a single molecule could induce this effect. To evaluate both of these possibilities, I generated antibody Fab fragments by digestion of complete antibody with papain (Figure 24A). FACS analysis revealed that these newly generated Fabs retained their ability to bind T cells (Figure 24B). In terms of clustering of BW, YAC and AB.1 T cells, I3/2 Fabs were as effective if not more so than the complete antibody at inducing clustering of these cells. These results suggest that cross-linking is not necessary to induce adhesion and that perhaps binding is either directly inducing some conformational switch in CD45 or perhaps blocking some other anti-adhesion interaction (Figure 24C). In contrast, MB23G2 induced clustering was completely lost in the Fab fragments, indicating that cross-linking of CD45 is necessary. This was confirmed when it was found that MB23G2 induced clustering could be restored if secondary cross-linking antibody towards the Fabs was added (Figure 24C). Together this further suggests that different anti-CD45 antibodies may act in different ways to induce adhesion responses in T cells.

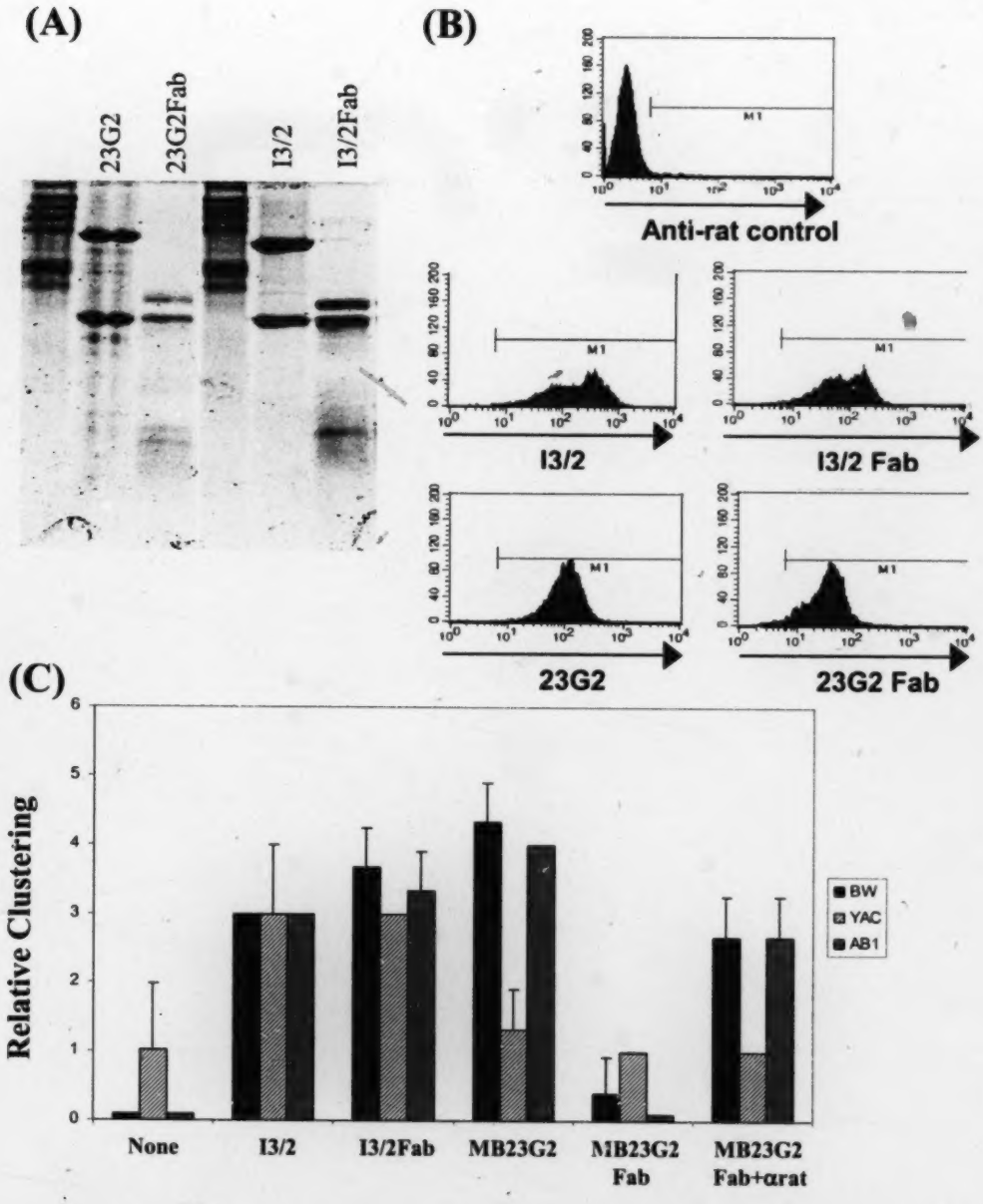


Figure 24: I3/2 Fabs, but not MB23G2 Fabs still induce clustering of T cells. Fab fragments of both I3/2 and MB23G2 were generated by digestion with papain (A), and as shown in (B) remained capable of binding the BW T cell line. Intact and Fab I3/2 and MB23G2 were then used to treat BW, YAC or AB1 T cells and clustering was quantified as described (C); additionally an anti-rat secondary antibody was used to cross-link Fab fragments in part (C). These results represent the average of three independent experiments.

4.5 Pan-CD45 antibody, but not anti-CD45RB antibody can induce clustering of phosphatase dead BW revertant cell line

The most likely explanation for any effects induced upon antibody binding to the extracellular domain of CD45 is that binding alters the phosphatase activity of this protein. To address this, I utilized a BW CD45 revertant cell line, which is lacking the majority of the cytoplasmic domain of CD45 including the D1 phosphatase domain resulting in the absence of phosphatase activity. Treatment of this cell line with I3/2 still induced clustering, although at a somewhat reduced level (figure 25). In contrast, MB23G2 had no effect on this revertant cell line, indicating that this antibody requires the CD45 cytoplasmic domain and likely its phosphatase activity in order to elicit its effects (Figure 25).

4.6 Homotypic T cell clustering is partially dependant on LFA-1

Previous work looking at clustering induced by soluble anti-CD45 antibodies indicated that this adhesion was mediated primarily through LFA-1 interactions with ICAM-1 [168] or ICAM-3 [167]. To determine whether this was also true of my observed adhesion, BW and AB.1 T cells were pre-incubated with anti-LFA-1 blocking antibody and then treated with I3/2 or MB23G2. In agreement with previous studies, I3/2 mediated adhesion appeared completely dependent on LFA-1 (Figure 26B). In contrast, 23G2 appeared only partially dependant on LFA-1 in BW cells at the 1 hour time point (Figure 26B); in AB.1 cells or BWs at later time points the MB23G2 clustering was relatively unaffected (Figure 26A and 26B). However, further blocking of this LFA-1 independent adhesion in AB.1 or BW was partially achieved by treatment with EDTA

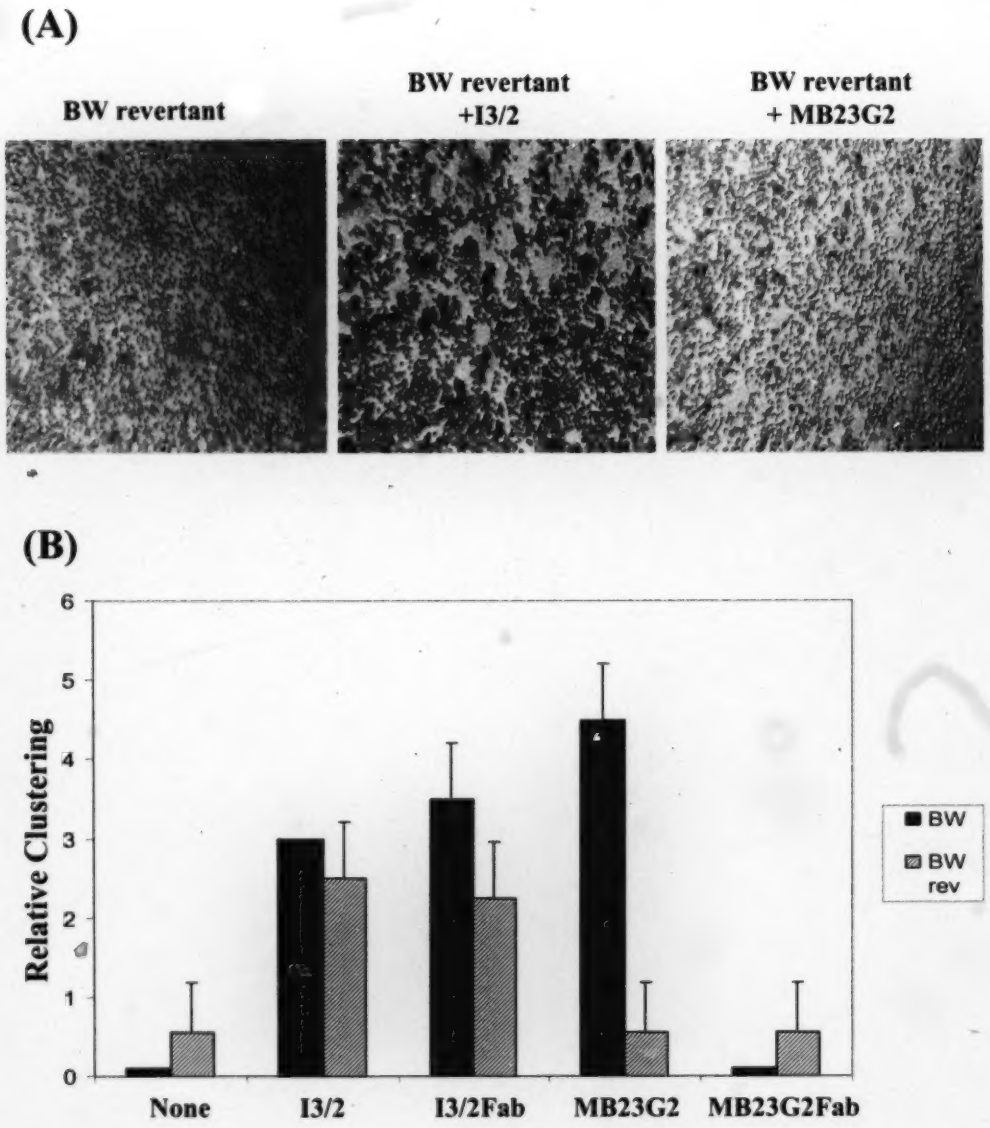


Figure 25: 23G22, but not I3/2 requires CD45 phosphatase activity to induce clustering. Intact and Fab versions of I3/2 and MB23G2 were used to treat wildtype BW and BW CD45 revertant (phosphatase dead) cell lines. Representative data displaying clustering of BW revertant cells by intact antibody is shown in (A). Clustering was subsequently quantified as described, and display graphically in (B). The results presented in (B) represent the average of two experiments.

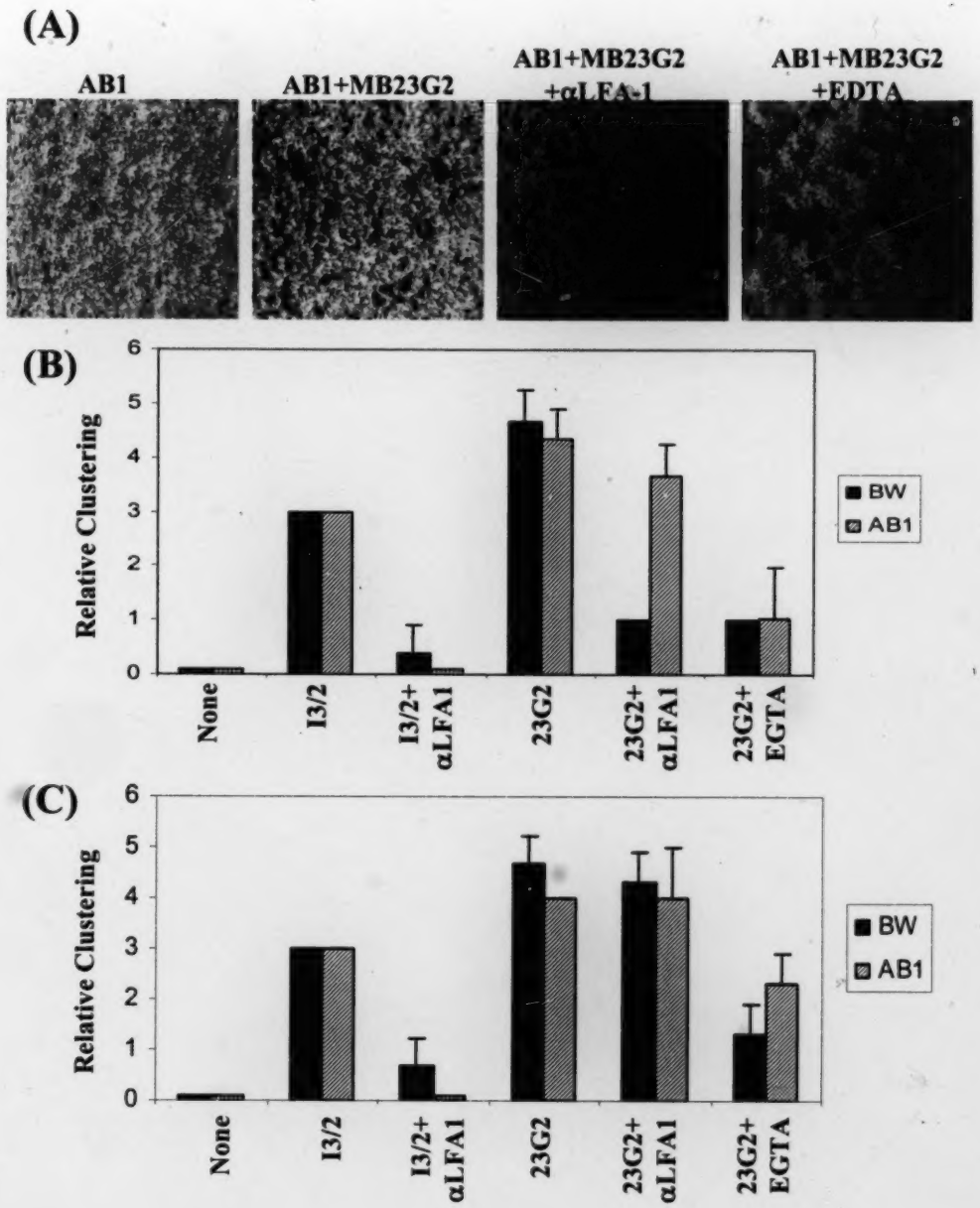


Figure 26: I3/2 induced clustering is LFA-1 dependant whereas MB23G2 clustering is only partially dependant on LFA-1. Prior to incubation with MB23G2 or I3/2 for 1hr (B) or 2hrs (C), BW and AB1 T cells were either pretreated with anti-LFA-1 antibody or left untreated. Clustering was then quantified as described. Presented is the average of three experiments. Additionally, (A) represents MB23G2 induced AB1 clustering following 1 hour with or without LFA-1 blocking antibody.

which suggests the involvement of other integrins, as integrin adhesion relies on available Mg^{2+} ions [66]. These results suggest that although I3/2 mediated adhesion is reliant on LFA-1, MB23G2 may act though multiple integrins including LFA-1.

4.7 Clustering requires kinase activity and the cytoskeleton

Given what had been observed thus far, it appeared as though binding of antibodies to the extracellular domain of CD45 initiated signaling that resulted in triggering of integrin adhesion. To begin to understand which pathways may be involved, I utilized a variety of inhibitors including bisindolymaleimide (bim1) (PKC), PP2 (Src-family kinases), Herbimycin A (protein tyrosine kinases), Sodium Vanadate (PTPase) and Cytochalasin E (actin cytoskeleton). Cells were pretreated with inhibitors prior to incubation with anti-CD45 antibodies. These results indicate that although both I3/2 and MB23G2 appear to act independently of PKC and the Src-family kinases, they did rely on some protein tyrosine kinases since Herbimycin A completely abrogated clustering (Figure 27). CD45 induced clustering was also significantly reduced by vanadate, which is known to block PTPase activity including that of CD45, therefore indicating that this phosphatase activity is also required for optimal clustering. Cytochlasin E also had a pronounced effect on the ability of both I3/2 and MB23G2 to induce homotypic T cell adhesion. Taken together this indicates that the signal induced by both antibodies are similar in that neither utilizes PKC or Lck to mediate adhesion, but require some balance of protein tyrosine kinase/phosphatase activity to transmit their signals in addition to requiring rearrangement of the cytoskeleton to mediate this adhesion.

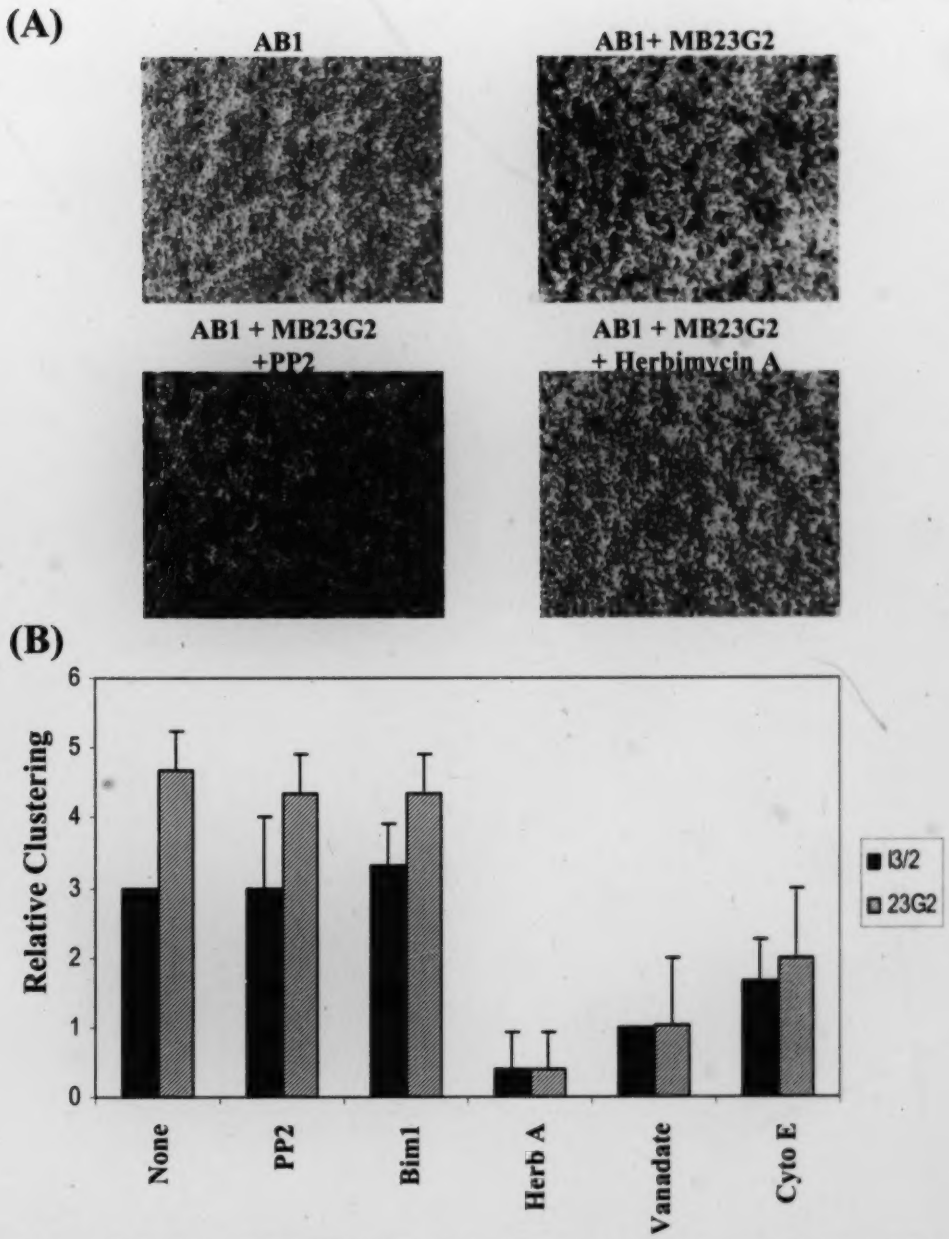


Figure 27: I3/2 and MB23G2 induced clustering requires kinase activity and the cytoskeleton. Prior to incubation of BW cells with anti-CD45 antibodies, cells were pretreated with the Src kinase inhibitor PP2, the PKC inhibitor bim1, a protein tyrosine kinase inhibitor Herbimycin A or cytochalasin E to disrupt the cytoskeleton. (A) Representative MB23G2 induced clustering of AB1 cells in the presence or absence of PP2 and herbimycin A. (B) Clustering was subsequently analyzed as described, and the data presented graphically as an average of three experiments.

4.8 Anti-CD45RB antibody can induce T cells to bind untriggered T cells

Another question I wanted to answer was whether the observed homotypic cell-cell adhesion requires both participating cells to be activated, or whether a triggered cell could bind to a non-triggered cell. To evaluate these two possibilities I mixed different ratios of CD45 positive and CD45 negative BWs and treated these cells with anti-CD45 antibodies to which only the CD45 positive cells could respond. The cells were allowed to cluster for one hour at which point I quantified what proportion of cells were clustered as compared to the original cell ratio. In I3/2 treated cells it appeared that only the CD45 positive cells were clustering, whereas MB23G2 treated cells actively clustered with CD45 positive or negative cells (Figure 28). This indicated that MB23G2 binding to CD45 triggers binding of T cells to other T cells regardless of whether they too have been triggered, whereas I3/2 cannot induce this same effect.

4.9 Pan-CD45 antibody enhances clustering in response to PMA

Given that I3/2 induces clustering as a Fab and does not require the cytoplasmic domain, this suggests that perhaps it may be acting to block some anti-adhesion role of the CD45 extracellular domain. To more closely study this possibility, I compared the effects of PMA on CD45 negative and CD45 positive cell lines. I expected that PMA which normally induces clustering, to cause stronger and/or more rapid adhesion in CD45 negative lines, and that addition of I3/2Fab may result in a similar degree of clustering in CD45 positive lines. BW cells were first treated with I3/2 Fab, followed by addition of PMA and clustering observed at specific times points. CD45 negative BW celis responded well to PMA with clustering observed by 30 minutes (Figure 29B). In contrast, CD45 positive BWs did not respond to PMA alone. However, pretreatment

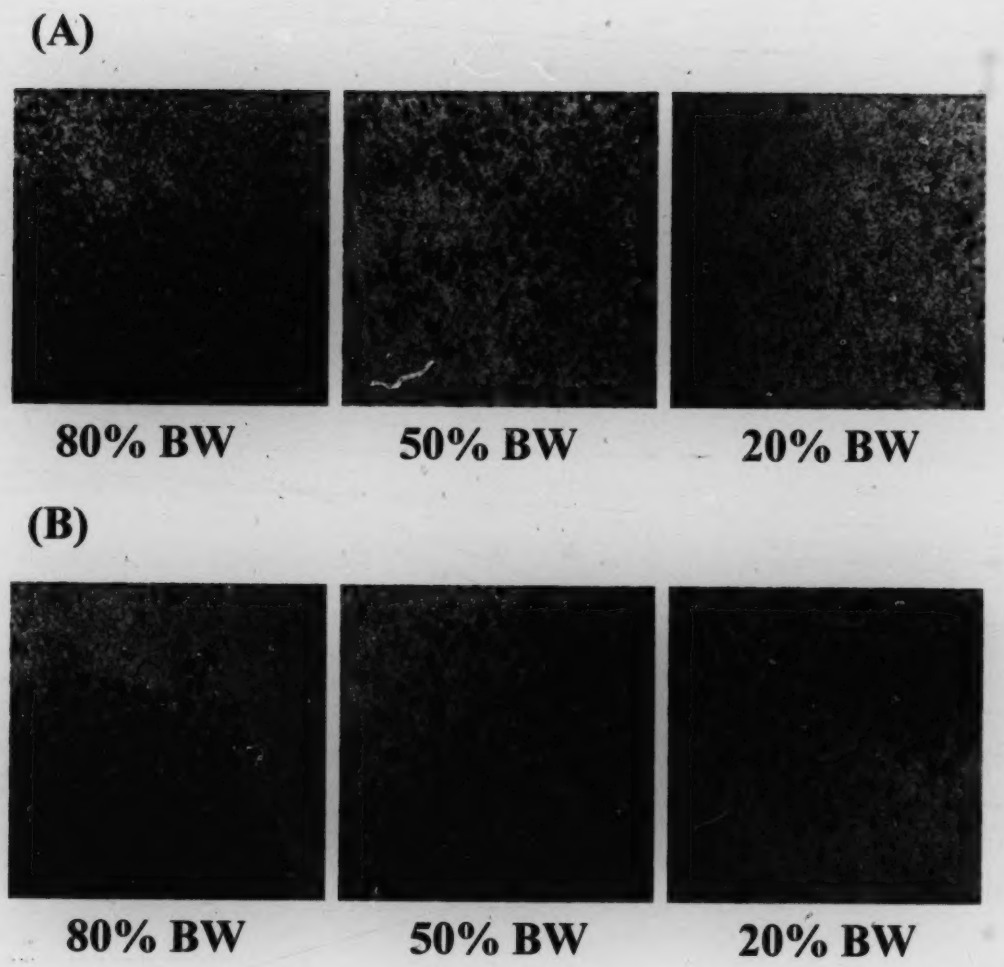


Figure 28: 23G2, but not I3/2 can induce T cells to adhere to untreated cells. CD45 positive and negative BW cells were mixed at specific ratios followed by treatment with soluble I3/2 (A) or MB23G2 (B) for 1 hour. This experiment was performed twice with similar results.

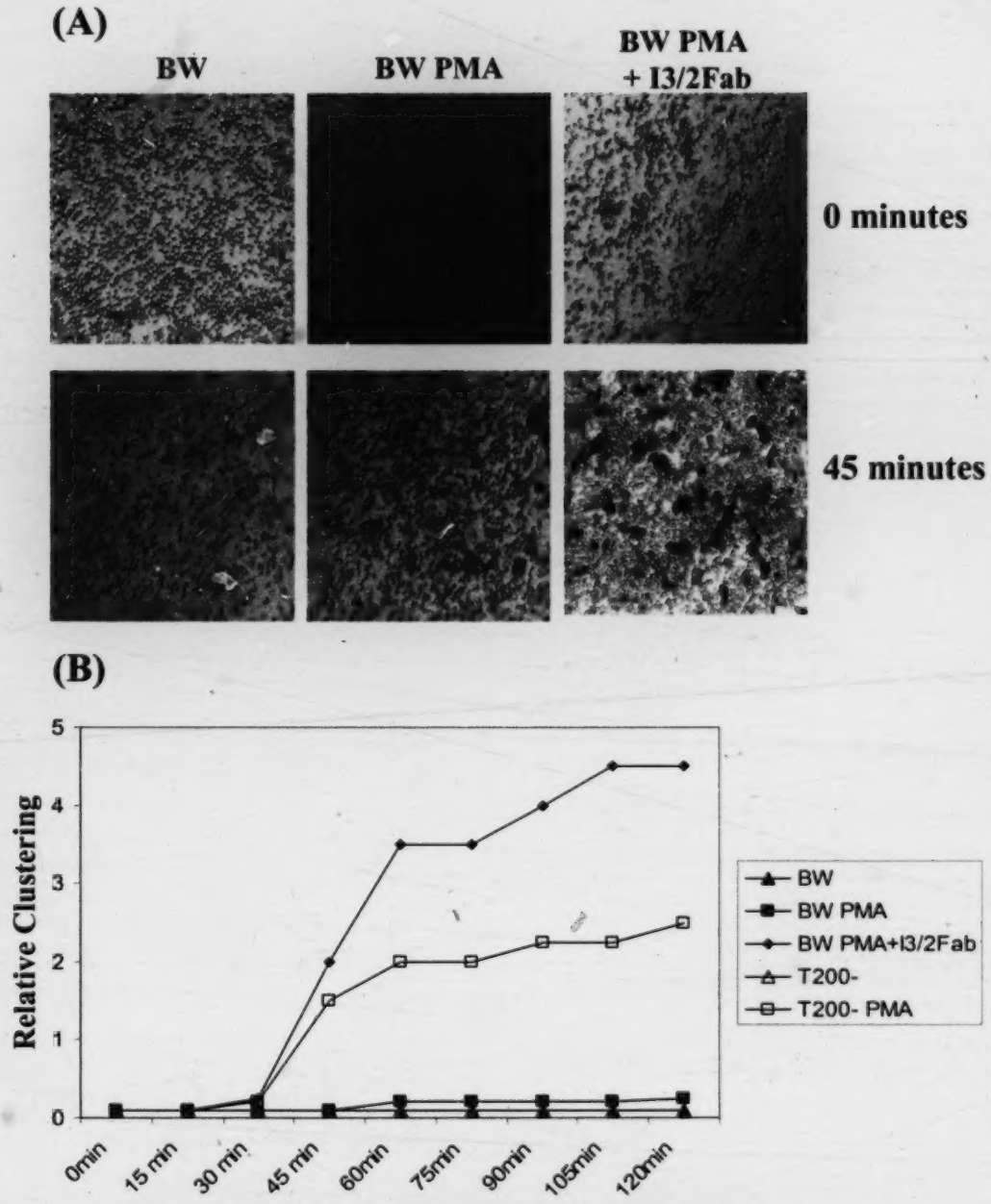


Figure 29: PMA induces clustering only in CD45 negative BWs or CD45 positive BWs treated with I3/2. CD45 positive and negative BW cells were incubated with PMA alone or together with I3/2 Fab for specific times. Representative data is shown in (A) for clustering of CD45 positive BW cells at 0 and 45 minutes. Clustering was subsequently quantified as discussed, these results are the average of two independent experiments (B).

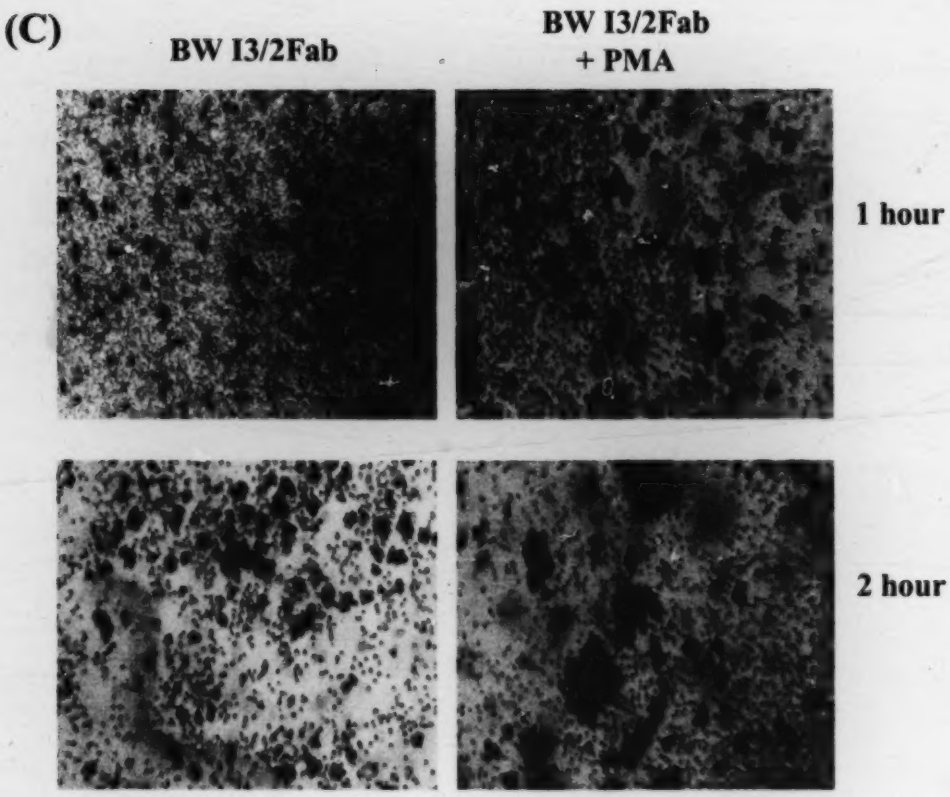


Figure 29: (C) Representative data comparing I3/2 Fab or I3/2 Fab+PMA induced clustering of BW cells 1 or 2 hours post-treatment.

with I3/2 Fab antibody plus PMA resulted in a very potent response (Figure 29A and 29B). Additionally, although these cells did not respond to PMA alone, however, when combined with I3/2Fab, this response was significantly stronger then that observed to I3/2 Fab alone (Figure 29C). This data suggests that the extracellular domain may play some role in blocking adhesion and that this effect can be effectively blocked by the anti-CD45 antibody I3/2.

4.10 Anti-CD45 antibodies trigger clustering of *ex vivo* T cells

All the data accumulated to this point has been demonstrated in cell lines or clones, and so it was next important to insure that these findings are also relevant *in vivo*. To determine its *in vivo* relevance, clustering of *ex vivo* T cells and thymocytes was evaluated in response to both I3/2 and MB23G2. As with cell lines, clustering occurred in both thymocytes and splenocytes following treatment with either I3/2 or MB23G2, however clusters took significantly longer to form, up to two hours and were much smaller than those seen in the cell lines (Figure 30). This might suggest that only specific subpopulations of cells are capable of responding to these antibodies, however at this time those specific populations remain unknown.

4.11 Summary

To summarize, the results presented here confirm that although different CD45 antibodies all bind essentially the same protein, the way in which the extracellular domain of CD45 is bound results in significantly different cellular responses. Binding of MB23G2 appears to crosslink CD45 and directly trigger some response in the cytoplasmic domain ultimately inducing strong cell-cell adhesion. In contrast, CD45 antibody I3/2 is superior at inducing adhesion to plastic when immobilized, but when

added in soluble form can trigger somewhat weaker cell clustering perhaps through blocking of some anti-adhesion interaction involving the extracellular domain of CD45.

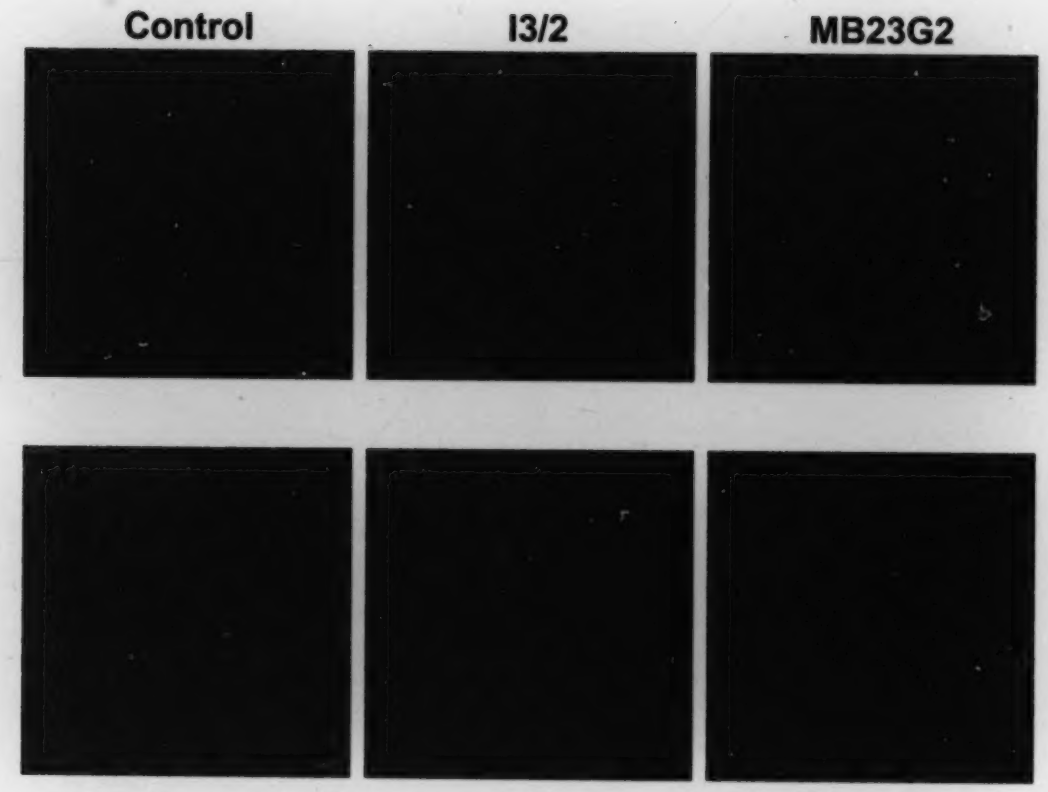


Figure 30: I3/2 and 23G2 induce clustering of *ex vivo* T cells and thymocytes. *Ex vivo* Splenocytes (A-C) or thymocytes (D-F) were isolated from C57B/6J mice and were left untreated (A, D) or were treated with I3/2(B, E) or MB23G2 (C, F) for 2 hours. This experiment was repeated three times with similar results.

CHAPTER V

DISCUSSION

5.1 Summary of Results

My original hypothesis proposed that regulation of CD45 on T cells during activation or development may occur through binding of ligands to the extracellular domain of CD45, and that these interactions would be primarily mediated through the conserved carbohydrate moieties present on the surface of this molecule. In order to gain understanding as to the identity of any potential CD45 ligands and what role their interaction may have, I approached this problem from two directions. First I characterized a variety of lectins present on professional APCs within the primary and secondary lymphoid organs, as this class of proteins is known to specifically recognize carbohydrate and so seemed fitting candidates for interacting with CD45. Second, I studied the effects of a number of CD45 specific monoclonal antibodies on T cells, in order to study the effects that binding of potential ligands may have in terms of regulation of this PTPase.

With regards to C-type lectin characterization, my data suggests that Dectin-1 is capable of binding T cells and in doing so may act to enhance activation in combination with sub-optimal triggering through the TCR, as indicated by increases in cytoskeletal rearrangements, degranulation and cytokine production. In terms of T cell development, Dectin-1 appears to strongly recognize more immature DN and DP thymocytes as well as $CD4^+$ SP thymocytes, while it displays significantly weaker binding $CD8^+$ SP thymocytes. This preference for $CD4^+$ T cells also exists in more mature T cells, as does

a preference for binding to T cells with activated or memory phenotypes. Through these studies I was also able to gain insight into the specificity of Dectin-1, in that my data suggests that Dectin-1 recognizes large negative sulfated ligands on T cells and that this specificity can be altered by removal or addition of divalent calcium ions. In contrast to Dectin-1, my data suggests that Dectin-2 does not appear to bind any significant proportion of thymocytes or T cells, even though multiple isoforms of this lectin are present in both the thymus and spleen. The third lectin I characterized, CIRE, is also present in both the spleen and thymus, and even though it does not appear to bind thymocytes or play any role in T cell development, my data does indicate that this lectin can bind naive and activated mature T cells and act as a potent co-stimulator. When bound in combination with anti-CD3 it could significantly augment all aspects of T cell activation assessed. Although I observed binding of both Dectin-1 and CIRE to T cells, this binding appeared to occur independently of CD45, however I cannot conclude that these lectins do not bind CD45, but only that ligands other than CD45 exist on these T cells.

The second part of this study looked at the effects of soluble anti-CD45 monoclonal antibodies on T cell clustering and adhesion, and attempted to dissect the mechanisms by which binding of CD45 elicits these effects. The results presented herein confirm that which has been previously suggested in that binding of antibodies to different CD45 epitopes result in different effects. However, my results also demonstrated distinct differences in the mechanism used by these antibodies to elicit these effects. I showed that the CD45RB specific antibody MB23G2 which potently induced clustering, appeared to require cross-linking of CD45 and that this resulted in signaling through the

cytoplasmic domain, activation of kinases and ultimately in triggering of multiple adhesion molecules including LFA-1. In contrast my results suggest that the pan-specific I3/2 antibody which more efficiently triggered adhesion, appeared to act independently of the cytoplasmic domain of CD45 and mediate adhesion through LFA-1 alone perhaps though blocking of some anti-adhesion property of CD45.

5.2 Characterization of T cell binding for Dectin-1, Dectin-2 and CIRE

Carbohydrate binding lectins and lectin-like receptors are known to play many important roles within the immune system. Through their binding to T cells, lectins such as the galectins, selectins, siglecs and C-type lectins are able to effectively modulate cellular activity, adhesion, homing and even death. Of particular relevance to this study are the numerous reports that have identified lectins selectively expressed on dendritic cells or other professional APCs. These cells play crucial roles in both the development of an appropriate T cell repertoire and in the initiation of primary immune responses. Although the function of many of these lectins has yet to be elucidated, it is now becoming apparent that many of these may serve multiple functions including T cell-APC interactions [210]. To date, the best characterized examples of lectins known to play such a role are DC-SIGN, Dectin-1 and DCAL-1, all of which have been observed to directly bind and augment T cell responses. [74, 76, 77].

5.2.1 Dectin-1 and CIRE bind T cells

When this study was first initiated I was primarily interested in what role lectin binding played in the context of T cell development and so I first determined that these lectins were in fact expressed on thymic stromal populations. Following this I next wanted to determine whether they actually had any significant role in development of

these cells or perhaps in regulation of mature T cell responses. However, before any precise functional role for Dectin-1, Dectin-2 or CIRE could be established, I first had to determine whether these lectins were even capable of binding thymocytes or mature T cells. For Dectin-1, previous reports had already established binding to numerous T cell lines, and that this binding was increased following T cell activation with ConA [75, 76]. My data supports these findings, but in addition I observed direct binding to both *ex vivo* thymocyte and T cell populations suggesting *in vivo* relevance. With regards to T cell development, my results indicate that Dectin-1 has highest affinity for more immature DN and DP thymocytes, in addition to $CD4^+$ SP thymocytes, whereas its ligand is apparently down-regulated on $CD8^+$ SP thymocytes. A similar trend is also true of mature T cells in that Dectin-1 displays superior binding to $CD4^+$ T cells, as compared to their $CD8^+$ counterparts. Interestingly, this correlates well with the original study by Ariizumi *et al.*, in that the majority of cell lines shown originally to bind Dectin-1 were derived from $CD4^+$ T cells [76]. In agreement with Dectin-1 preferentially binding activated cell lines, I also found that this lectin bound with highest affinity to *ex vivo* T cells with activated or memory phenotypes as compared to naive T cells; suggesting involvement of this lectin following the initiation of an immune response.

In terms of Dectin-2, my data suggests that this lectin does not bind any of the tested T cell populations to any significant extent, and for this reason I opted not to characterize it any further. However, recent reports suggest that Dectin-2 may in fact bind regulatory T cells and may be actively involved in both the induction and maintenance of peripheral tolerance [193]. I suspect the reason for not detecting this bound population in the conjugate assay system employed in my studies was that it did

not have the required sensitivity needed to detect this relatively minor peripheral T cell population, which constitutes approximately only 5% of peripheral T cells [211].

In contrast to Dectin-2, my results show for the first time that CIRE does in fact bind T cells, but unlike Dectin-1 is unable to recognize thymocytes. I show here that in addition to T cell lines, CIRE also binds *ex vivo* T cells, with an apparent preference for those with an activated phenotype. Similar to Dectin-1, CIRE does display a slight preference for $CD4^+$ T cells, however this prejudice is less extreme than that of Dectin-1. Also, when compared to Dectin-1, binding of soluble CIRE appears to be of significantly lower affinity. However, *in vivo* mechanisms including triggered clustering, help from other adhesion molecules or perhaps oligomerization, as has been shown for the closely related DC-SIGN molecule, may be involved in strengthening interactions involving CIRE [171].

When analyzing the binding of either CIRE or Dectin-1, it is immediately apparent that binding of these lectins is highly dependant on both the developmental and differentiation state of the T cell. As previously discussed, lectins are often associated with recognition of specific carbohydrates, and correspondingly, it has long been appreciated that cell-surface glycosylation changes with T cell development and activation [212, 213]. The most conceptually straightforward regulatory mechanism to explain this is via the up- or down-regulation of the ligand itself or of relevant glycosylation enzymes including glycosyltransferases and glycosidases, whose expression, interestingly, is also tightly regulated in a developmentally and activation dependant manner [210, 212, 214-217].

In studying CIRE and Dectin-1 binding to T cells, I had also originally intended to identify what, if any specific roles the different isoforms of these lectins may play. However the results presented here suggest that in terms of affinity for T cells, both isoforms of Dectin-1 and CIRE are not significantly different. However, *in vivo* alternative splicing may affect properties such as oligomerization or stability, as is the case for lectins such as DC-SIGN [218].

5.2.2 Requirements of lectin binding and potential ligands

One of the primary requirements for typical C-type lectin binding to its carbohydrate ligand is the coordination of one or more calcium ions. In fact structural analysis of a variety of lectins has now revealed remarkable similarity among different CRDs with respect to ligand recognition in that that the primary protein-monosaccharide interactions are often directly mediated by Ca^{2+}, with only a few other accessory interactions being involved [171, 219]. However, analysis of the CTLD of Dectin-1 (Figure 4) reveals that some of these residues crucial for coordination of divalent calcium ions are absent. It was therefore not surprising that when studied with regards to yeast binding Dectin-1 was found to bind in a calcium independent manner [179, 186]. However, in contrast to the situation with yeast, my data indicates that binding to T cells does require Ca^{2+} and that other divalent metal ions such as Mg^{2+} cannot substitute. This finding would suggest that although some of the Dectin-1 calcium binding residues are divergent, enough are still intact to allow for coordination of this metal ion. One possibility, is that the remaining residues within the primary site (site#2) (Figure 4) are still capable of binding calcium, which would then participate directly in carbohydrate binding; or alternatively that calcium coordinated within this site, or the intact site #3

(auxiliary site) would alter the confirmation of the Dectin-1 CTLD, allowing for stable recognition of its corresponding ligand. With regards to CIRE, little work has been done to characterize its binding, however initial inspection of its CRD reveals that all the residues necessary for calcium binding are present (Figure 4). It is for this reason that I was surprised to find that chelation of divalent metal ions including calcium had no effect on T cell binding, suggesting that these ions are not necessary for stable binding of its corresponding ligand. However, it is also possible that CIRE is capable of coordinating calcium and that this calcium is just not necessary for binding its ligand on T cells. Alternatively, it may be that the calcium is so tightly coordinated that chelators such as EDTA and EGTA cannot gain access, to in order to remove it. The idea that CIRE does in fact coordinate calcium is supported by the observation that folding of this lectin occurs much more efficiently in the presence of excess calcium following purification from *E. coli* (Appendix Figure 2).

As most lectin and lectin-like receptors bind carbohydrate ligands, I was also interested in determining whether this was also true of Dectin-1 and CIRE. Unfortunately, because CIRE generally displayed quite weak binding to most T cells it was not possible to draw any firm conclusions as to which carbohydrates significantly reduced binding. With regards to Dectin-1, previous studies indicated that Dectin-1 binds a protein ligand(s) as binding was effectively blocked by treatment of T cells with trypsin. This study also suggested binding was independent of carbohydrate based on two findings: first that there was no observed lectin blocking using a panel of five monosaccharides; second that pretreatment of cells with tunicamycin, which blocks glycosylation of newly synthesized proteins, only had a minimal effect on Dectin-1

binding. However this tunicamycin treatment also had relatively little effect on binding of other *bone fide* lectin controls [76]. In contrast, my initial screens of Dectin-1 revealed that its binding was potently inhibited by fucoidin, a large negatively charged sulfate sugar composed primarily of repeating disaccharide units of fucose. Although I was unable to refine its specificity through the use of the mono- and oligosaccharides components that comprise fucoidin, subsequent screening demonstrated that Dectin-1 appears to bind numerous large negative sulfate sugars including fucoidin, dextran sulfate, heparin, haparan sulfate and chondroitin sulfate A and C. However other related sugars such as chondroitin sulfate B and hyaluronate were not able to block, suggesting there is some degree of specificity to Dectin-1 ligand recognition, although the details regarding its specificity have yet to be determined [220]. Further evidence for Dectin-1 binding a sulfated proteoglycan ligand on T cells came from the observation that cells lacking surface sulfation through blockade with chlorate, displayed significantly reduced binding to Dectin-1. Interestingly, specific recognition of sulfated carbohydrates by lectins is quite common, particularly among the selectins which are often associated with lymphocyte homing [221, 222].

Previous studies looking at Dectin-1 binding had also suggested that binding of this lectin to yeast and T cells may utilize two separate binding sites based on the observation that laminarin could only block binding to yeast and not T cells [75]. However my data clearly indicates that binding to yeast is also blocked by fucoidin, and that this inhibition is similar in magnitude to that observed for laminarin. This would suggest that Dectin-1 may utilize a single binding site, or at least some common feature for both T cell and yeast recognition. This raised the question as to how a single binding

site could recognize two such divergent ligands? The key to this appears to depend on the presence or absence of calcium as I observed that beta-glucans such as laminarin can readily occupy the binding site of Dectin-1 in the presence or absence of calcium. However, sulfated ligands including fucoidin require calcium coordination in order to stably bind Dectin-1, perhaps due to the direct association of the positive metal ion with the negative sulfate residues or some other common feature present on these carbohydrates. Alternatively, binding of calcium may alter the confirmation of the CTLD in such a way as to allow for stable recognition of this sulfated ligand, possibly by allowing other electrostatic interactions to occur between the CTLD and the sulfate residue. Regardless, this may provide the first example of differential ligand specificity by lectins based on their coordination of divalent metal ions.

The purpose for which this study was originally initiated, however, was to determine whether these lectins were capable of binding the extracellular domain of CD45 on T cells and in doing so modulate its activity. My initial results from Dectin-1 and CIRE binding to CD45 positive and negative T cell lines indicated that both lectins are capable of binding T cells in the absence of CD45, and in many cases binding actually appeared enhanced. Furthermore, in lectin cross-linking experiments no proteins were pulled-down in the size range expected for CD45 by either lectin. However, it is important to emphasize that these results do not rule out the possibility that these lectins are capable of binding CD45, they only indicate that they are capable of binding ligands other than CD45 on the surface of T cells. It is possible that, due to the variable glycoform and isoform usage of CD45, under the conditions used during cross-linking I do not see significant binding. Interestingly, although I did not observe binding of lectins

to CD45, analysis of the CRD of CIRE would suggest it binds mannose and Dectin-1 was found to bind sulfated sugars, both of which have been observed to be displayed on CD45 glycans [154, 223].

With regards to the ligands recognized by Dectin-1 and CIRE on the surface of T cells, cross-linking experiments with Dectin-1 were unable to pull-down any specific binding partners, which may be related to its binding of sulfated carbohydrates. Sulfate modifications and additions such as heparin sulfate, for which Dectin-1 was found to bind, are found on all eukaryotic cells in various forms and so due to their abundance, it is likely that Dectin-1 may not bind one or two specific ligands, but instead recognize a number of different sulfated ligands. In fact a number of lectins and other proteins, such as the selectins, which are capable of recognizing sulfated ligands have been observed to bind multiple targets, all of which are bound only when properly post-translationally modified [224]. As a result, Dectin-1 binding to T cells may be indirectly controlled through regulation of different sulfotransferases or other glycosylation machinery in the T cell which have been shown to vary depending on the differentiation state of the cell [225]. Additionally, interactions between sulfated fucans such as heparin sulfate and proteins have already been implicated in many biological processes including cell adhesion, regulation of growth and proliferation and activation [225]. In contrast to Dectin-1, my results for CIRE suggest that it may recognize a single protein of 80-85kDa that remains unidentified.

5.2.3 Lectin binding to other lymphocyte populations

In addition to binding of T cells, my data indicates that Dectin-1 is capable of binding both NK cells and B cells, whereas CIRE exhibits stronger binding to B cells

than to T cells. Similar to T cells, lectins binding to, and found on, the surface of NK and B cells have been observed to play numerous roles with regards to their function including homing, cell-cell adhesion and regulation of cellular activity and proliferation [226-231]. Given that Dectin-1 has been observed to bind sulfated ligands, it was not surprising to discover that it was quite non-specific in terms of the cell types bound, as all of these would be expected to have an abundance of sulfated proteoglycans on their surfaces. In contrast, because CIRE appears to have a more restricted ligand specificity, the fact that it binds B cells in addition to T cells may suggest that these cells express a related ligand and that binding of CIRE to these cells would likely elicit similar effects. However, whether the binding of Dectin-1 and CIRE serves purely to mediate adhesion or may perhaps act to directly modulate cellular activity is still a matter of speculation until more work can be done to study these interactions on these other lymphocyte populations.

5.2.4 Summary

To briefly summarize this section, although Dectin-2 was not observed to bind T cells, both Dectin-1 and CIRE were capable of binding multiple T cell populations in addition other types of lymphocytes. Dectin-1 was quite promiscuous in terms of its binding, likely due to its recognition of sulfated carbohydrates, which are found on the surface of nearly all cells. However, Dectin-1 did display some preference for immature thymocytes, $CD4^+$ cells and T cells with an activated phenotype. Also of interest was the discovery that the addition or removal of calcium from Dectin-1 could essentially modify its specificity. CIRE was also shown to bind both mature T and B cells although at considerably lower affinity than Dectin-1, and although the specificity of this lectin has

yet to be determined, initial evidence suggests that its ligand specificity is considerably more restricted than that of Dectin-1.

5.2.5 Future Directions

Future directions for this section would include further characterization of the carbohydrate specificity of CIRE, perhaps through the attachment of an N-terminal biotin tag to CIRE and subsequent binding to streptavidin for tetramer studies allowing for higher avidity interactions. It would also be of great interest to isolate and identify the 85kDa protein found to bind CIRE, as this might provide clues as to the role CIRE plays in APC-T cell interactions. In contrast to many C-type lectins, my data suggested that CIRE may bind T cells in the absence of calcium, however analysis of the CIRE CRD would suggest that this lectin is capable of binding this and perhaps other divalent metal cations. Studies using radiolabeled calcium may be able to conclusively determine whether or not the lectin does in fact coordinate calcium. Although through these studies I was able to determine that Dectin-1 recognizes sulfated carbohydrates, the sugars found to block this lectin had no single common structural determinant. Thus, studies could also be undertaken to further refine the specificity of this lectin, although it is also possible that no one determinant exists and that binding relies on the correct positioning of multiple factors. To this end, isolation and characterization of one or perhaps more of the Dectin-1 ligands on T cells may provide additional clues as to how this lectin recognizes its cognate ligand(s).

5.3 Lectin binding and Functional relevance

Lectin binding to lymphocytes is associated with a wide variety of functional outcomes, for example binding of selectins is often associated with homing to lymphoid

organs and sites of infection, the galectins have been observed to induce or protect cells from apoptosis and the siglecs are capable of mediating cell-cell adhesion or suppressing activation of B cells [227, 230, 231]. Of particular relevance to this study are the number of lectins that have been demonstrated to be important in the context of T cell-APC interactions. DC-SIGN is the best characterized example of such a lectin, and has been shown to be important in mediating initial APC-T cell adhesion, however DCAL-1 and Dectin-1 have also both been suggested to possess co-stimulatory activity [74, 76, 77]. As previously discussed, my data clearly demonstrates that both CIRE and Dectin-1 are in fact capable of binding multiple T cell and/or thymocyte populations, leading to the obvious question: What functional role does this binding serve in the context of T cell activation and/or development?

5.3.1 Role of Dectin-1 and CIRE in T cell regulation and development

My data clearly suggests that both CIRE and Dectin-1 are capable of augmenting T cell responses in the presence of sub-optimal stimulation through the TCR, the simplest interpretation of which is that these lectins possess co-stimulatory activity as has already been suggested for Dectin-1 [76, 174]. However it is important to state that cell-surface molecules that improve the adhesion of T cells without directly modulating signaling cannot be regarded as co-stimulatory even though increased adhesion often enhances T cell activation [64]. By allowing for more efficient T-cell-APC conjugation, adhesion molecules such as LFA-1 have been observed to lower the threshold of antigen necessary for T cell activation by close to 100-fold, however it does not effect activation otherwise; whereas co-stimulatory molecules including CD28 not only reduce the amount of

engaged TCRs necessary for activation but also allow activation to lower affinity ligands by signaling through pathways independent of the TCR [232].

If we first consider Dectin-1, previous reports have suggested that this lectin possesses co-stimulatory activity, as when combined with sub-optimal stimulation through the TCR it enhanced proliferation, cytokine production and lead to upregulation of specific cell-surface activation markers [76, 174]. Although my results confirm these findings, in that Dectin-1 was observed to enhance IFN-γ production, cell spreading and degranulation of CTL when co-immobilized with anti-CD3, other data gathered may suggest that Dectin-1 operates in more of an adhesion capacity and that these results may be due to increased binding of and thus signaling through the TCR. The first piece of evidence that may suggest more of an adhesion role for Dectin-1 is that unlike established co-stimulators or CIRE which are capable of potently enhancing signaling and T cell activation when combined with even very low levels of stimulation through the TCR; Dectin-1 only modestly increased activation and was often unable to completely restore activation to optimal levels when combined with reduced levels of anti-CD3. Furthermore, Dectin-1 appeared to have no effect on T cell activation when levels of anti-CD3 were below that which was capable of eliciting some effect on its own [64]. Additionally, I demonstrated that even soluble monomeric Dectin-1 appeared to display very strong binding to many of the tested T cell lines and *ex vivo* populations suggesting a relatively high affinity interaction with one or more ligands on these cells. However, many co-stimulators and even the TCR itself bind with very low affinities (K_d between 10^{-4} and 10^{-6}) and for this reason often rely on increased avidity binding or the help of separate adhesion molecules which display significantly stronger adhesion (K_d around 10^{-}

^{6} or 10^{-7}) in order to maintain stable binding to their corresponding ligands [233-237]. In fact high affinity TCRs are specifically selected against during thymocyte development, due to the fact that if these or other molecules with the capacity to stimulate cells were capable of readily binding their targets, this would likely lead to aberrant T cell activation and the risk of autoimmunity developing [17]. However, given the strong binding demonstrated by Dectin-1 to T cells, it is entirely possible that when co-immobilized with anti-CD3 it could increase adherence of T cells allowing for more efficient signaling through the TCR complex as compared to in the absence of Dectin-1. Lastly, it is uncharacteristic of co-stimulatory molecules to display the lack of specificity observed for Dectin-1. Its ability to bind virtually every lymphocyte population evaluated and its binding to sulfated carbohydrates potentially present on a variety proteoglycans would suggest that Dectin-1 may be capable of recognizing a number of ligands further arguing against any role as a specific T cell co-stimulatory protein. Although, it should also be mentioned that at this time no conclusive evidence exists to rule out the possibility that Dectin-1 does possess some co-stimulatory activity. Regardless of its specific mechanism, both co-stimulatory and adhesion molecules play crucial roles in regulation of T cells and are necessary for proper T cell development and responses.

In contrast, CIRE potently increased all aspects of T cell activation even at very low levels of stimulation through the TCR. Interestingly, in a soluble monomeric form it showed significantly lower affinity for T cells as compared to Dectin-1 and this was especially true for AB.1 CTL clones on which the functional experiments were carried out. This low affinity is not only consistent with that expected for a co-stimulatory molecule, but also argues against any purely adhesive role, as CIRE-T cell binding would

likely contribute little to T cell adhesion to plastic when immobilized, and would therefore not be expected to significantly increase signaling though the TCR complex alone.

With regards to a role in T cell development and/or activation *in vivo* for these two lectins, because of the ability of Dectin-1 to recognize multiple thymocyte populations together with its expression in the thymus this would strongly implicate this lectin as having some role in T cell development. Although its precise role in this process has yet to be elucidated, given that it recognizes T cells with relatively high affinity it could be important as an adhesion molecule in directing interactions between specific thymic stromal DCs and developing thymocytes necessary for their selection or further differentiation. When one considers that Dectin-1 is specifically expressed on DCs, which are primarily implicated in negative selection, and that its binding appears to augment T cell responses; taken together this may suggest that it could play a direct and significant role in negative selection, perhaps through altering the threshold of TCR activation necessary to pass this selective process. In terms of T cell responses, like many co-stimulatory and adhesion molecules, Dectin-1 is up-regulated following DC activation [172, 173]. Together with data demonstrating its ability to bind and help activate $CD4^+$ and $CD8^+$ T cells, this would suggest that following activation of DC in the periphery, these cells would up-regulate Dectin-1 together with other cell-surface molecules and migrate to the secondary lymphoid organs where Dectin-1 would likely act to help initiate an adaptive immune response [76, 174]. Additionally, its preferential recognition of activated and memory T cells would suggest that Dectin-1 may be important later in immune responses perhaps in regulating the activity or number of

effector T cells generated or in the formation or subsequent activation of memory T cells during secondary responses. The ability of CIRE to bind similar populations and significantly enhance T cell activation would suggest a similar role for this lectin in terms of mediating T cell responses. However, somewhat surprisingly, in contrast to many other co-stimulatory molecules CIRE appears down-regulated upon DC activation [195]. However it is also possible that under some conditions such as those used by Caminschi *et al* to stimulate DCs, CIRE expression is down regulated, whereas under different conditions its expression may by maintained of even upregulated allowing this lectin to be involved in the initiation or maintence of specific adaptive immune responses [195]. The down-regulation of CIRE following DC maturation may also suggest a potential role in antigen uptake, as these receptors are often most highly expressed on immature DC in the periphery. Interestingly, other receptors including DC-SIGN, which serve dual roles in both Antigen uptake and T cell-DC interaction have displayed similar patterns of expression and are down-regulated following DC activation [210].

5.3.2 Future directions

Although my data indicated the ultimate functional outcome of CIRE and Dectin-1 binding to T cells was the augmentation of signals provided through the TCR; the precise mechanism though which these lectins elicit these effects remains to be elucidated. Based on my results, it appears that with regards to T cells, CIRE may act in a truly co-stimulatory fashion and important clues as to its role and mechanism could be provided through the identification of its ligand and the signaling molecules activated following engagement with its T cell ligand. In contrast, I suspect that Dectin-1 may act in more of an adhesion capacity, but similar to CIRE, further study of its ligand(s) and the

pathways initiated following its binding could reveal whether it acts to trigger separate pathways or merely enhance signaling through enhancement of TCR binding.

My results clearly indicate that both lectins, when combined with anti-CD3 could enhance activation of CTL clones. However, in order to directly establish a role *in vivo*, study of CIRE and Dectin-1 binding to *ex vivo* populations is also necessary. Eventually, the generation of tools such as lectin knockout mice will even make *in vivo* study of these lectins possible. Also, although Dectin-1 was observed to bind thymocytes, the effects of this binding and any potential role in T cell development remains to be established. This could be accomplished through the study of lectin binding to *ex vivo* populations or perhaps the use of fetal thymic organ cultures combined with specific blocking antibodies, carbohydrates or lectin antagonists.

Another potential project to come from this study would be to study the effects of CIRE and Dectin-1 binding to NK or B cells. Given what has been observed on T cells, it is possible that binding of these lectins may have similar stimulatory effects on these other lymphocyte populations; for example the priming of NK cells or even polyclonal activation of B cells by DCs under specific conditions. Although this remains speculation at this point, the ability of these lectins to bind other lymphocyte populations would suggest that they may act to modulate multiple components of both the innate and adaptive immune systems.

5.4 CD45 antibody binding and T cell responses

As no specific CD45 ligands have been identified, another approach I utilized in order to determine what the effects ligand binding to the extracellular domain of CD45 may have, was to use monoclonal anti-CD45 antibodies to mimic ligand binding. Other

reports using a similar approach with immobilized or soluble anti-CD45 antibodies alone found that binding of CD45 could elicit significant cytoskeletal rearrangements and cell-cell adhesion respectively [165, 167-169]. Interestingly, many discrepancies exist between these studies depending on the anti-CD45 monoclonal antibody used to trigger these cellular effects, leading to the suggestion that binding of different CD45 molecules or even binding of the same CD45 molecule in different ways may trigger alternate pathways resulting in different cellular effects. In the current study I have directly compared the effects and mechanisms used by several different anti-CD45 antibodies and found that different anti-CD45 antibodies do in fact differ in their ability to induce specific cellular effects and that this likely results from utilization of different mechanisms by these antibodies. Furthermore, this study allowed us to gain some insight into how soluble anti-CD45 treatment can result in homotypic T cell-T cell adhesion, a phenomenon that although previously described, had not been well characterized [167-169].

5.4.1 Clustering versus spreading

Due to the fact that cytoskeletal rearrangements and cell-cell adhesion have both been observed following CD45 treatment and that both are easily observable, I chose to evaluate these two effects when comparing binding of different anti-CD45 antibodies. My results indicate that different anti-CD45 antibodies, although all capable of binding essentially the same molecule, exhibit qualitative and quantitative difference in the outcomes they elicit. Whereas the pan-specific CD45 antibody I3/2 is superior at inducing cell spreading when immobilized, binding of the CD45 antibodies M1/9, MB23G2 and MB4B4 result in more rapid and intense homotypic cell clustering; and

some antibodies such as M1/89 have no effect at all when bound. These findings lend support to the hypothesis that binding of antibodies to different epitopes of CD45 acts to bring forth different effects. Furthermore these results can be extended to potentially suggest that binding of different ligands to CD45 may display similar differences in terms of the cellular effects they elicit. Although specific ligands remain to be identified, if similar differences were found to exist, then differential ligand specificity conferred though alternative CD45 isoform or glycoform usage could be a potential mechanism by which to control CD45 activity and thus regulate T cells. However these findings also raise another important question, being how could the binding of different antibodies or potential ligands to CD45 mechanistically act to elicit such qualitatively or quantitatively different responses?

5.4.2 Mechanism of anti-CD45 effects

The ability of antibodies to CD45 to induce the reported effects may reflect their ability to induce conformational changes within the cytoplasmic domain of CD45 that directly affect its enzymatic activity, or alter associations with proteins that physically interact with CD45. Alternatively binding of antibodies to the extracellular domain of CD45 may simply act to dimerize this PTPase which has been suggested to modulate its activity or perhaps act to block interactions between CD45 and other proteins both on the surface of the same or other cells that ultimately influence the regulation of different T cell properties. Due to the fact that the antibodies I3/2 and MB23G2 displayed the most significant differences in terms of their effects when bound to T cells, I opted to further characterize the mechanism of these two antibodies.

If we first consider the pan-specific anti-CD45 antibody I3/2, my data suggests that binding of this antibody may be acting to block some anti-adhesive property or interaction in which the extracellular domain of CD45 participates. Experiments using monovalent antibody Fab fragments of I3/2 were still able to induce cell-cell adhesion, even though these antibodies were unable to crosslink this PTPase. Although this is suggestive of a blocking role, it is also possible that binding of a single Fab could still potentially induce some conformational change in CD45 altering its activity. Arguing against this second possibility was the observation that I3/2 induced clustering in the BW revertant cell line in which the majority of the CD45 cytoplasmic domain was absent including its D1 phosphatase domain. With regards to signaling, src-family kinases including Lck, which are known to rely on CD45 phosphatase activity for their full activation, have also been observed to play an important role in anti-CD45 antibody mediated cytoskeletal rearrangements [105, 165]. Given these observations, Lck would seem a fitting downstream candidate for CD45 mediated clustering, however inhibition of this and other Src-family kinases with PP2 had no significant effects on cell-cell adhesion. Clustering was shown to rely on the activity of some kinases, as the general protein tyrosine kinase inhibitor herbamycin A completely abrogated adhesion. Likewise, the phosphatase inhibitor vanadate also significantly reduced clustering. However one intrinsic complication when looking at signaling and cell-cell adhesion that was encountered by mine and previous studies is trying to separate signaling that is occurring downstream of CD45 and that which may be associated with activation of adhesion molecules such as integrins [238, 239]. Previous studies had originally characterized anti-CD45 induced heterotypic T cell-macrophage adhesion as utilizing

cAMP/cGMP and PKA dependant pathways [238], however it was later discovered that this signaling was occurring in monocytes following LFA-1 engagement to ICAM-1 [239]. Similar to that reported in previous studies, my results also suggest that this I3/2 mediated adhesion occurs via LFA-1. Given that I3/2 antibody binding does not directly appear to induce signaling, one intriguing possibility is that binding of this antibody may block some anti-adhesive property of the extracellular domain of CD45. This is not the first time this has been suggested, in fact a previous study suggested that not only may CD45 possess such an activity, but that alternate isoforms may differ with regards to this property [240]. One potential mechanism to describe how this could occur relates to the abundant presentation of negatively charged carbohydrates such as sialic acid on CD45. Due to the large size of this molecule and its overall negative charge, cells presenting appropriate iso- or glycoforms of this molecule could potentially repel each other prior to other adhesion molecules having the chance to interact. In fact, one study looking at T cell-B cell conjugation found that adhesion could be significantly enhanced following removal of sialic acid by neuraminidase treatment [241, 242]. Taken together, I suggest a model for I3/2 mediated adhesion which suggests that I3/2 binding may mask specific negatively charged carbohydrates presented by CD45 which otherwise act to repel cells carrying a similar charge (Figure 31). Once blocked, other adhesion molecules, primarily LFA-1 act to mediate adhesion between T cells. Additional evidence to support this model comes from the observation that PMA treatment, which normally induces cellular clustering, was unable to do so in the BW T cell line unless pretreated with intact or I3/2 Fab fragments; however it was able to induce homotypic clustering alone in a CD45 negative BW cell line. It is also interesting to note that in general, when grown in cell

culture many CD45 negative cells lines including BW, YAC and SAKRs are more prone to forming small clusters suggesting that these cells appear to display more adherent properties. However it should also be noted that these are not targeted knock-out cell lines and so other alterations may be present to account for this adhesion. Although it is possible that *in vivo* binding of a ligand to CD45 may have a similar effect as I3/2 binding. It is also possible that if alternative iso- or glycoforms of CD45 differ with respect to their intrinsic anti-adhesive properties, as has been suggested, and through regulation of CD45, T cells could effectively regulate their basal adhesiveness.

In contrast to I3/2, MB23G2 appears to utilize a more direct approach in mediating homotypic T cell adhesion (Figure 31). My results demonstrate that in order for MB23G2 to trigger cell clustering it had to crosslink the extracellular domain and in doing so mediated some signal through the cytoplasmic domain, potentially through the phosphatase activity of CD45. Unlike I3/2, MB23G2 was also capable of activating cells to bind both non-activated and other triggered cells suggesting the direct activation of some adhesion molecule(s), which were capable of recognizing a ligand on both populations. Although MB23G2 mediated adhesion appeared to be partially dependant on LFA-1, this only accounted for a portion of the observed clustering, as when blocked with specific anti-LFA-1 antibodies, significant clustering could still occur. Given that EDTA could further reduce blocking, this may implicate integrins other then LFA-1 in this adhesion as integrins require Mg^{2+} ions for stable binding [66]. In terms of the specific signaling pathways utilized downstream of CD45 binding, my results suggest that this occurs independently of Src-family kinases and PKC, however does require the activity of some other kinases and phosphatase in addition to the cytoskeleton. However

dissection of the specific pathways is made difficult for the same reasons as described for I3/2.

So although the specific mechanism of action for these two antibodies remains to be determined, particularly in terms of signaling, this study does highlight the fact that binding of monoclonal antibodies to different CD45 epitopes does elicit very different responses through the activation of distinct mechanisms. Interestingly, when either antibody was used to treat *ex vivo* T cell or thymocyte populations, only small discrete clusters were observed to form and these took significantly longer to appear as compared to clustering of cell lines. This likely suggests that within a heterogeneous population, only specific sub-groups of T cells or thymocytes are even capable of responding. Taken together this illustrates how utilization of specific iso- or glycoforms of CD45 on different T cell populations may confer specificity to specific ligands or alter other properties of its extracellular domain in such a way as to allow these T cells to potentially modulate their own activity or responses.

5.4.3 Future directions

Dissection of the pathways used following triggering of CD45 with antibodies has been difficult, particularly in the case of cell-cell adhesion due to the simultaneous signaling occurring downstream of adhesion molecules such as LFA-1. Potential clues may come from future studies looking at adhesion of T cells to immobilized CD45 where adhesion molecules do not have available ligands to bind and therefore will not signal. Previous studies report that much signaling occurring within T cells following plating on immobilized anti-CD45 is mediated though Lck and leads to phosphorylation of two proteins involved in formation of focal adhesions: paxillin and Pyk2 [165, 166].

Although the finding that signaling occurs through Lck conflicts with the data presented here, alternative pathways may also be involved. One clue may have come from an observation made by Tara Lysechko (personal communication) in our lab that suggests that following engagement of CD45 with plate bound antibody, it was found to associate with CD3-zeta, a critical signaling component known to lay upstream of many proteins including those involved with cytoskeletal rearrangements and adhesion. In fact it has previously reported that not only can CD3-zeta be found associated with CD45, but is also a high affinity target for its phosphatase activity [243]. Within recent years it has also become apparent that multiple populations of CD3-zeta exist within T lymphocytes. These include a normally non-cytoskeletal associated form that becomes phosphorylated by Src-family kinases and may associate with the cytoskeleton following TCR engagement, and a form that is always found associated with the cytoskeleton termed cska-zeta (cytoskeleton associated-zeta) which is phosphoryated independently of Src-family kinases [244, 245]. Whether these two populations have different roles in T cell activation or potentially downstream of CD45 induced signaling is still not understood. However some preliminary results do suggest a role for some population of CD3-zeta in CD45 triggered adhesion as experiments using BW cells, which normally lack CD3 zeta, made to express CD25/CD3-zeta chimeric proteins show significantly enhanced responses to immobilized CD3 as compared to those expressing CD25 and CD25/CD3 epsilon controls constructs (Appendix figure 3).

5.5 Overall Conclusions

Although I was unable to establish a definite role for Dectin-1, Dectin-2 and CIRE in binding to CD45, both Dectin-1 and CIRE did demonstrate that ability to

recognize multiple T cell and/or thymocyte populations, in addition to other lymphocyte populations. Moreover, these lectins exhibited the ability to enhance T cell activation when combined with sub-optimal stimulation through the TCR complex. Taken together with the pattern of expression on professional APCs within both primary and secondary lymphoid organs, this suggests that these lectins could potentially play significant roles, not only in the development of T cells, but also in the initiation and maintenance of adaptive immune responses. Although much work is still required in order to elucidate the specific ligands and pathways utilized by these lectins during T cell recognition, the present study also revealed some clues, particularly for Dectin-1, with regards to its specificity for endogenous T cell ligands. My results suggest, for the first time, that a single lectin may be capable of recognizing multiple diverse ligands depending on the coordination of appropriate divalent metal ions.

It is also important to note that the data presented here cannot exclude the possibility that these lectins are able to recognize some isoforms of CD45, only that the lectins are capable of binding independently of CD45. Although a specific CD45 ligand still remains to be identified, data presented here using specific anti-CD45 antibodies suggests that binding of different CD45 molecules or even binding of different epitopes within the same CD45 molecule may act to elicit different effects. This would provide the cell a way to modulate its own activity through alteration of CD45 iso- or glycoforms thus conferring specificity to different ligands.

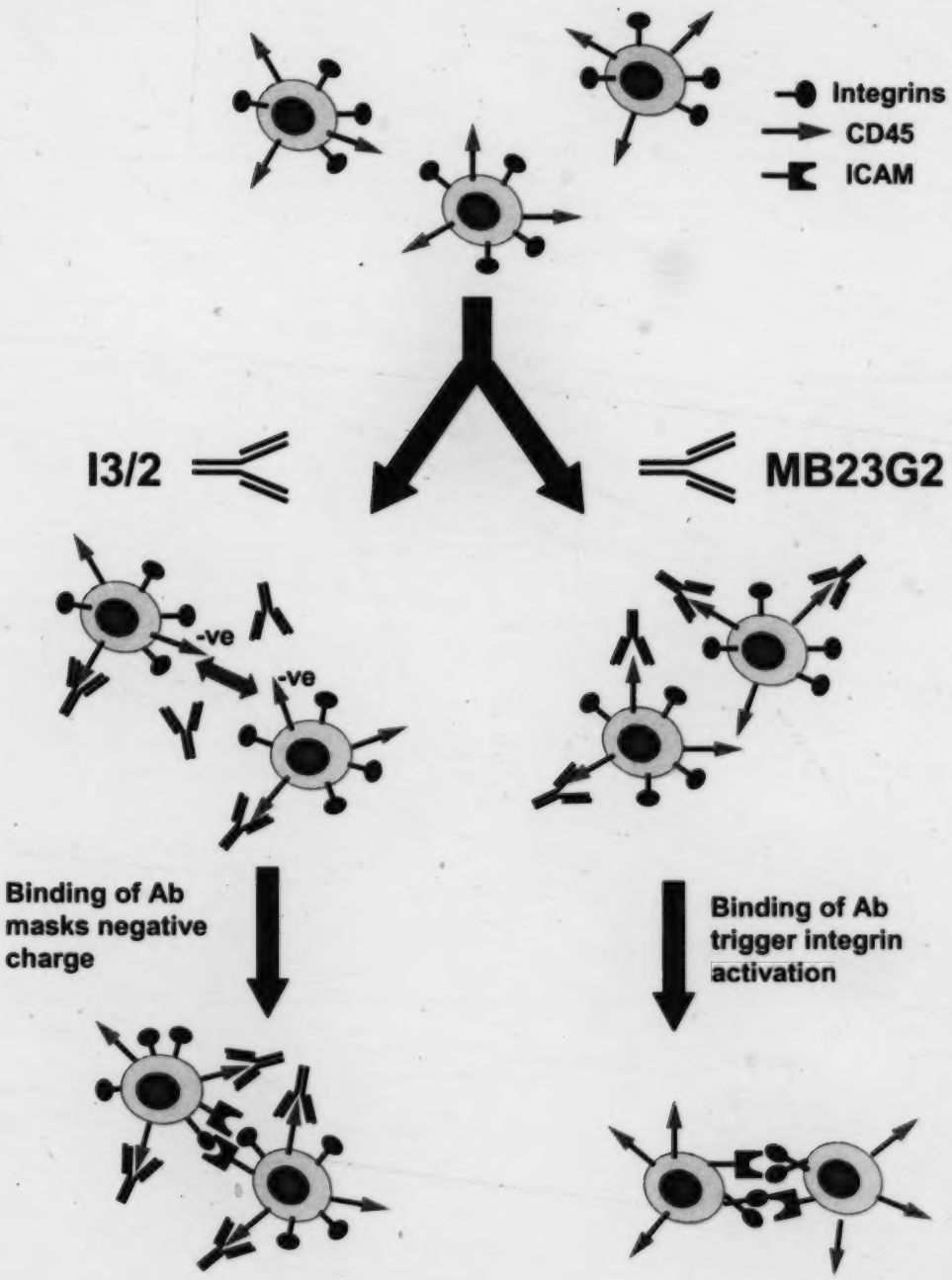


Figure 31:Model for Anti-CD45 induced clustering.

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APPENDIX

A.1 Dectin-1 and CIRE folding occurs more efficiently in the presence of Ca^{2+} ions.

Following purification of recombinant Dectin-1 and CIRE from bacterial lysates, the lectins were refolded under a variety of conditions prior to dialysis against PBS or TBS (Appendix figure 2C). Protein remaining in solution following dialysis was then separated by SDS-PAGE in order to evaluate efficiency of folding. Soluble Dectin-1 and CIRE are displayed in Appendix Figure 1B and 1C respectively. These results suggest that folding occurs more efficiently in the presence of divalent metal cations.

A.2 Expression of CD3-ζ allows for more efficient responses to immobilized anti-CD45

BW cells expressing CD25, CD25/CD3-ε or CD25/CD3-ζ constructs (appendix figure 3A) were stimulated on immobilized anti-CD25 or anti-CD45 for 45 minutes. Responses to antibody were then evaluated by calculation of percentage of cell spread which is presented graphically in appendix figure 3B, and represents the average of four experiments.

(A)

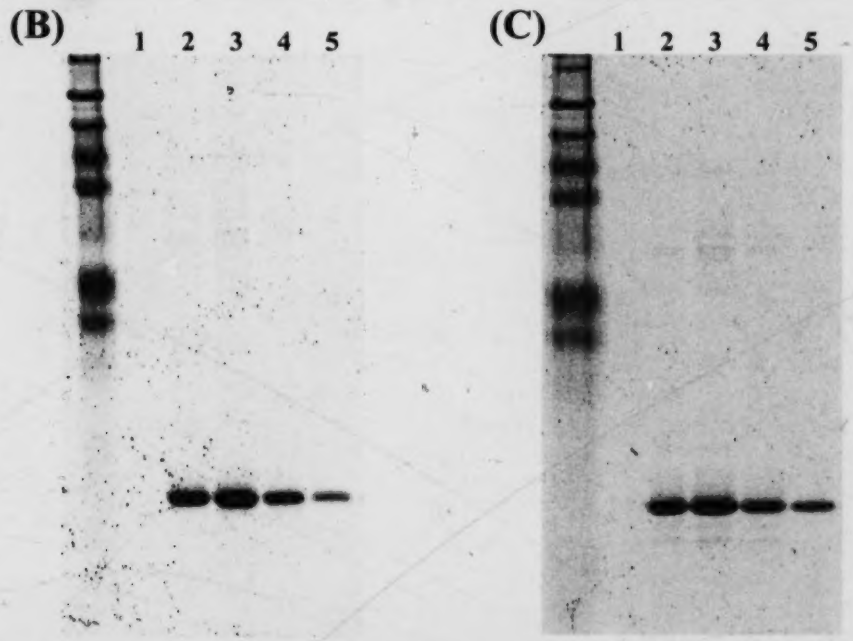
1→ PBS

2→ 50mM Tris (pH 8.0), 250mM NaCl, 10mM KCl, 500mM L-Arginine, 1mM EDTA, 10mM reduced Glutathione, 1mM oxidized Glutathione.

3→ 50mM Tris (pH 8.0), 250mM NaCl, 10mM KCl, 500mM L-Arginine, **2.5mM $CaCl_2$**, 2.5mM $MgCl_2$, 10mM reduced Glutathione, 1mM oxidized Glutathione.

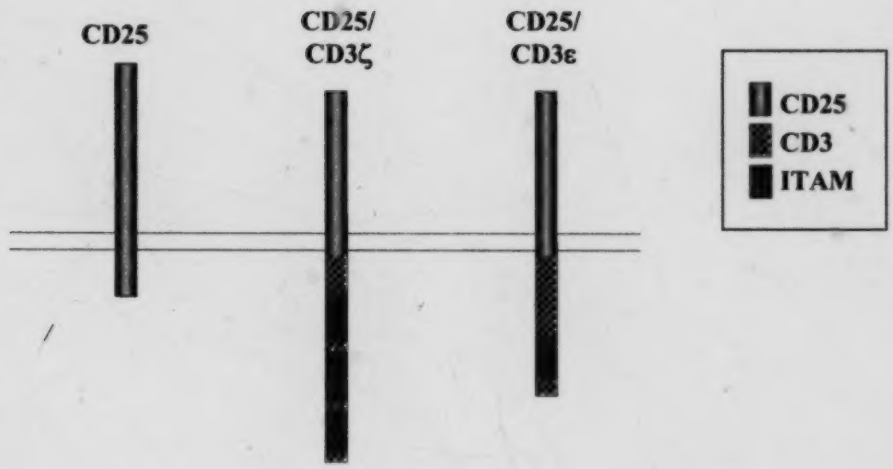
4→ 50mM Tris (pH 8.0), 10mM NaCl, 1mM KCl, 500mM L-Arginine, **2.5mM $CaCl_2$**, 2.5mM $MgCl_2$, 10mM reduced Glutathione, 1mM oxidized Glutathione.

5→ 50mM Tris (pH 8.0), 10mM NaCl, 1mM KCl, 500mM L-Arginine, 1mM EDTA, 10mM reduced Glutathione, 1mM oxidized Glutathione.

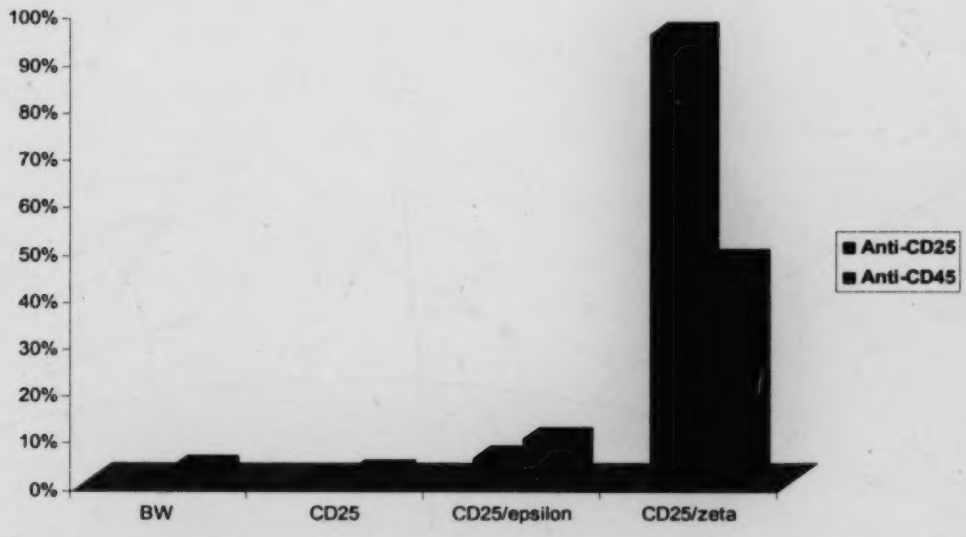


Appendix Figure 1: Lectin Folding occurs more efficiently in the presence of Divalent metal ions. Dectin-1 (B) and CIRE (C) purified from bacterial lysates were refolded under a number of conditions followed by dialysis against PBS and soluble protein was subsequently separated by SDS-PAGE and coomassie stained. Conditions used in refolding are shown in (A).

(A)



(B)



Appendix Figure 2: CD3-ζ is required for efficient responses to anti-CD45. Control BW or those expressing CD25, CD25/CD3-ε or CD25/CD3-ζ were stimulated on anti-CD25 or anti-CD45 for 45 minutes (A). Percent cells spread in response to antibody is presented graphically in (B) and represents the average of four experiments.